Defence Science Journal, Vol. 57, No. 5, September 2007, pp. 707-720

Protective Effect of Quercetin Against Sulphur Mustard-induced Oxidative Stress in Mice

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ABSTRACT

Sulphur mustard (SM) is a chemical warfare agent that causes serious blisters upon contact with human skin. SM alkylates DNA and several other macromolecules, and also induces oxidative stress. Quercetin, a bioflavonoid has wide pharmacological actions. The protective efficacy of quercetin (100 mg/kg, i.p. and 200 mg/kg, i.p.) was studied by administering three doses in mice against SM. The first dose was administered at 30 min prior, simultaneous, 2 h post or 24 h post, and two more doses on the next two days. SM was administered (in PEG 300) percutaneously at varying doses for survival and protection studies. SM was also administered at a dose of 2 LD_{50} (19.3 mg/kg) with and without quercetin treatment and various biochemical markers were estimated 7 days after SM administration. Histological examinations of vital organs were also carried out. The animals administered with SM died at various days depending upon the dose. The body weight decreased significantly. Quercetin protected the mice significantly, in a dose-dependent manner. The protection was better when the first dose of quercetin administered was 30 min prior or simultaneously. A significant decrease in reduced as well as oxidised glutathione and an increase in malondialdehyde, WBC count, RBC count, and haemoglobin were observed with 2 LD_{50} SM. Quercetin at 100 mg/kg and 200 mg/kg doses significantly protected the biochemical markers when the first dose of quercetin administered was 30 min prior or as simultaneous treatment. The histological lesions induced by sulphur mustard on liver, spleen, and skin were also significantly protected by quercetin when the first dose was administered 30 min prior or as simultaneous treatment. The present study shows that percutaneous administration of SM induces oxidative stress and quercetin can protect it as a prophylactic agent.

Keywords: Sulphur mustard, quercetin, oxidative stress, glutathione, malondialdehyde, DNA damage, prophylactic agent, chemical warfare agent

1. INTRODUCTION

The organophosphorus nerve agents and the blistering agents continue to be threats not only as chemical warfare agents, but also from the terrorist organisations. Though the Chemical Weapons Convention is signed and ratified by several countries and the stockpiled chemical warfare agents are being destroyed, still the threat persists from the use of chemical weapons. The mechanism of action of the nerve agents is clearly understood and effective and accepted treatment protocols are known[1]. In spite of research over several decades, no satisfactory prophylactic or treatment regimen has evolved for sulphur mustard (SM), a well known blistering agent. So the search for a better antidote is being pursued the world over.

Received 3 January 2006, revised 13 June 2006

The SM, commonly known as mustard gas, is chemically bis (2-chloroethyl) sulphide and an alkylating agent that causes serious blisters upon contact with human skin. SM has been used as a chemical warfare agent in many instances[2-5]. SM forms sulphonium ion in the body and alkylates DNA leading to DNA strand breaks and cell death[6]. Due to the high electrophilic property of the sulphonium ion, SM binds to a variety of cellular macromolecules[7]. Eyes, skin and the respiratory tract are the principal target organs of SM toxicity[6,8].

Antidotes to SM can act by four different mechanisms:

(a) Prevention of SM from entering the system (personal decontamination at the site of contact),

(b) Prevention of SM from alkylating critical target molecules mainly DNA,

(c) Retrieval of SM alkylated DNA

(d) Prevention and reversal of the cascade of secondary biochemical reactions of alkylation[6-9].

Several antidotes have been reported for reducing the systemic toxicity of SM in experimental animals[9-15]. Most of the antidotes are in various stages of development and so far none recommended. One of the interesting findings is that percutaneously administered SM is more toxic than subcutaneous and oral routes of administrations[16]. The most effective way of minimising SM toxicity is by decontamination either by physical adsorption or by chemical decontamination[17].

One of the important mechanisms of action of SM cytotoxicity is based on the depletion of reduced glutathione (GSH), and subsequent lipid peroxidation and free radical generation[7,18]. Flavonoids are reported to exhibit a wide variety of biological effects, including antioxidant and free radical scavenging activities. Quercetin and other flavonoids have been shown to modify eicosanoid biosynthesis (antiprostanoid and anti-inflammatory responses), protect low-density lipoprotein from oxidation (prevent atherosclerotic plaque formation), prevent platelet aggregation (antithrombic effects), and promote relaxation of cardiovascular smooth muscle (antihypertensive, antiarrhythmic effects). In addition, flavonoids have been shown to have antiviral and carcinostatic properties[19]. Ethanol-induced oxidative stress was protected by pre-treatment with quercetin by directly quenching lipid peroxides and indirectly by enhancing the production of the endogenous antioxidant GSH[20,21]. Protective effect of quercetin on carbon tetrachloride-induced hepatotoxicity, cyclosporin-induced nephrotoxicity, and cisplatin-induced cytotoxicity in cultured tubular epithelial cells have been reported[22-24]. Quercetin was effective in protecting silica-induced cell injury due to its antiradical property[25]. The study was aimed to evaluate the protective effect of quercetin against percutaneously administered SM.

2. MATERIALS AND METHODS

2.1 Chemicals

Quercetin dihydrate was purchased from Aldrich Chemical Company (USA). SM was synthesised in the declared facility of the Establishment and was found to be above 99 per cent pure by gas chromatographic analysis. All other chemicals used were of analytical grade.

2.2 Animals

Randomly bred Swiss female mice (25 g to 30 g body weight) maintained in the Establishment's animal house were used for the study. The animals were housed in polypropylene cages on dust-free rice husk as the bedding material. The animals were provided with pellet diet (Amrut Ltd, India) and water *ad libitum*. This study was approved by the Ethical Committee of the Establishment. A day before dermal application of SM, the hair on the back of mice were closely clipped using a pair of scissors.

2.3 Protection Studies on Animal Mortality

The SM was applied in varying doses on groups of mice and quercetin was administered intraperitoneally (i.p.) as three injections with the first injection given at 30 min prior, simultaneous (0 min), 2 h post or 24 h post. The remaining two doses of quercetin were given on the next two days. Two doses of quercetin used were 100 mg/kg and 200 mg/kg by

dissolving in polyethylene glycol (PEG 300). SM was diluted in PEG 300 and applied on the back of mice. The dilutions were made in such a manner that the quantity applied was not more than 100 µl. The animals were weighed daily and observed for mortality for a period of 14 days. Each group consisted of four mice per dose, and 3 to 4 log doses were used.

In another study, mice were administered with one intraperitoneal injection of quercetin (100 mg/kg and 200 mg/kg), either 30 min prior or simultaneous, and various doses of SM were applied percutaneously to find the effect of a single injection as prophylactic dose.

2.4 Protection Studies on Biochemical Variables

To evaluate the protective effect of quercetin on the GSH, GSSG, MDA, and DNA fragmentation, the following groups were kept:

I.	Distilled water, i.p.	+	PEG 300 dermal (control group)
II.	PEG 300, i.p.	+	SM (19.3 mg.kg^{-1}) in PEG 300 dermal (LD_{50} of SM = 9.7 mg/kg; SM group)
III.	Quercetin (100 mg/kg, i.p.) 30 min prior SM	+	SM (19.3 mg.kg^{-1}) in PEG 300 dermal
IV.	Quercetin (200 mg/kg, i.p.) 30 min prior SM	+	SM (19.3 mg.kg^{-1}) in PEG 300 dermal
V.	Quercetin (100 mg/kg, i.p.) simultaneously with SM	+	SM (19.3 mg.kg^{-1}) in PEG 300 dermal
VI.	Quercetin (200 mg/kg, i.p.) simultaneously with SM	+	SM (19.3 mg.kg^{-1}) in PEG 300 dermal
VII.	Quercetin (100 mg/kg, i.p.) 2 h post SM	+	SM (19.3 mg.kg^{-1}) in PEG 300 dermal
VIII.	Quercetin (200 mg/kg, i.p.) 2 h post SM	+	SM (19.3 mg.kg^{-1}) in PEG 300 dermal
IX.	Quercetin (100 mg/kg, i.p.) 24 h post SM	+	SM (19.3 mg.kg^{-1}) in PEG 300 dermal
X.	Quercetin (200 mg/kg, i.p.) 24 h post SM	+	SM (19.3 mg.kg^{-1}) in PEG 300 dermal

Three injections of quercetin were administered intraperitoneally, with the first injection given at 30 min prior, treatment simultaneous (0 min), 2 h post or 24 h post-treatment. The remaining two doses were given on the next two days. SM was diluted in PEG 300 and applied on the back of mice. The dilutions were made in such a manner that the quantity applied was not more than 0.1 ml. Blood was withdrawn from ocular plexus under ether anaesthesia and the animals were sacrificed by cervical dislocation, seven days after SM application. Each group consisted of four mice. The blood was used for the measurement of RBC and WBC counts and haemoglobin estimation. Pieces of liver were removed, blotted, weighed and used for the estimation of GSH, GSSG, and MDA. Pieces of liver, spleen and skin (on the site of SM application) were also removed for light microscopic studies.

The fluorometric method of Hisin and Hilf[26] was used for the determination of hepatic GSH, and GSSG concentration. For this, 150 mg of liver tissue was homogenised in 4 ml of phosphate EDTA buffer and metaphosphoric acid (25 %). The content of the tube was centrifuged and the supernatant was used for the estimation of GSH and GSSG. Hepatic lipid peroxidation was determined by measuring the level of MDA according to the method of Buege and Aust[27]. One hundred milligram of liver was directly homogenised in 5 ml of thiobarbituric acid reagent and boiled for 30 min. The contents of the tube were cooled, centrifuged, and absorbance of the clear supernatant was measured at 535 nm. The amount of MDA formed was calculated using a molar extinction coefficient of 1.58 x 10^5/M per cm. DNA fragmentation assay was carried out as per the method of McConkey[28], *et al*. Briefly, the frozen liver samples were homogenised in ice cold lysis buffer (10 mM tris, 20 mM EDTA, 0.5 % triton-x-100, p*H* 8) and then centrifuged at 27,000 g for 30 min. Both pellet (intact chromatin) and supernatant (DNA fragments) were assayed for DNA content, using diphenylamine[29]. The percentage of fragmented DNA was defined as the ratio of DNA content of supernatant at 27,000 g to the total DNA in the lysate[30]. The other haematological variables, viz., RBC and WBC counts and blood haemoglobin were analysed using Beckman Coulter Cell Counter (USA).

2.5 Protection Studies on Histological Lesions

The excised skin portion was removed and stretched in the wax tray and immersed in 10 per

cent neutral buffered formalin (p*H* 7.2) for 4 h. After proper hardening, the skin tissue was chopped into small pieces and fixed in fixative for another 24 h. Hepatic and splenic tissues were removed, pruned, blotted, and weighed. These tissues were fixed in 10 per cent buffered formalin. After proper fixation, small pieces of the tissues were processed by dehydration and embedded in paraffin wax. Multiple sections from each block were prepared of 5-6 μm thickness and stained with hematoxylin and eosin for microscopic observation[31]. From each tissue 10 slides were prepared. Out of the 60 slides (6 animals), six slides were selected for lesion evaluation. From each of the selected slides, 90 μm^2 area was identified randomly and lesions were marked and compared with that of control. The severity of lesions was characterised using LEICA-QWIN-500 image analyser and converted into percentage. The lesions were observed under microscope, characterised and scored using LEICA-QWIN-500-IW image analyser.

2.6 Statistical Analysis

LD_{50} and confidence intervals were determined by the moving average method[32]. If the confidence intervals could not be determined due to per cent and 100 per cent mortality in subsequent doses, then the doses producing 0 per cent and 100 per cent mortality were taken as lower and upper limits of confidence interval, respectively. The biochemical parameters were analysed by one-way ANOVA with Dunnett's multiple comparisons procedure. A probability of < 0.05 is taken as statistically significant. SigmaStat (SPSS Inc, USA) was used for the statistical analyses.

3. RESULTS

The animals administered with SM died at various days depending upon the dose. The LD_{50} of SM by percutaneous route was found to be 9.7 mg/kg. Quercetin protected the mice significantly, in a dose-dependent manner. The protection was seen only when quercetin was given either as 30 min prior or simultaneous treatment. Single dose as well as three doses given in these groups protected the mice and the protection was more or less similar (Table 1).

The body weight of mice administered with 19.3 mg/kg (2 LD_{50}) decreased significantly compared to the control group. Quercetin protected the decrease in body weight significantly, in a dose-dependent manner. The protection was better when the first dose of quercetin administered was 30 min prior or simultaneous. Significant protection was observed when quercetin was given either as a single dose or as three doses (Table 2).

The control group values of reduced glutathione was found to be 3.50 ± 0.10 μ moles/g of liver, oxidised glutathione was found to be 1.86 ± 0.33 μ moles/g of liver, malondialdehyde level was found to be 3.86 ± 0.10 μ moles/g of liver. There was a significant decrease in GSH as well as GSSG content and an increase in MDA following SM application (Table 3). Quercetin 100 mg/kg and 200 mg/kg doses significantly protected

Table 1. Protective effect of quercetin against percutaneously administered sulphur mustard

Group (First dose of quercetin)	Quercetin 100 mg/kg LD_{50} (mg/kg) (confidence limit)	PI	Quercetin 200 mg/kg LD_{50} (mg/kg) (confidence limit)	PI	No. of doses of quercetin
SM only	9.7 (6.1 - 15.2)	-	-	-	-
– 30 min + SM	19.3 (7.3 - 51.4)	2.0	38.6 (14.8 - 100.3)	4.0	3
0 min + SM	19.3 (10.6 - 35.3)	2.0	78.0 (27.6 - 220.1)	8.0	3
+ 2 h + SM	9.7 (5.9 - 15.8)	1.0	9.7 (5.9 - 15.8)	1.0	3
+ 24 h + SM	8.1 (0.2 - 12.4)	0.8	8.1 (0.2 - 12.4)	0.8	3
– 30 min + SM	38.6 (22.2 - 67.3)	4.0	78.0 (27.6 - 220.1)	8.0	1
0 min + SM	30.7 (21.6 - 43.5)	3.2	54.6 (15.2 - 196.5)	5.6	1

Protection Index (PI) = Ratio of LD_{50} with treatment to LD_{50} without treatment

the decrease in GSH and GSSG, and an increase in MDA induced by SM, when the first dose of quercetin administered was 30 min prior or simultaneous. The percent DNA fragmentation was increased following SM application and quercetin at a dose of 200 mg/kg, significantly protected the DNA damage when it was administered 30 min prior or simultaneous (Table 3).

There was no significant change in the WBC count after SM administration. Significant increase in RBC count and haemoglobin content was observed after percutaneous administration of SM. Quercetin administered as either 30 min pre-treatment or as simultaneous treatment significantly protected them (Table 4).

Table 2 . Percent change in body weight, seven days after 2 LD_{50} of percutaneously administered SM and protection by quercetin

Group (First dose of quercetin)	Quercetin 3 doses (i.p.)	100 mg/kg 1 dose	Quercetin 3 doses (i.p.)	200 mg/kg 1 dose
Control	98.7 ± 1.3	-	-	-
SM only	58.7 ± 1.5^a	-	-	-
– 30 min + SM	91.1 ± 3.8^b	68.2 ± 3.4^a	94.0 ± 3.1^b	89.1 ± 5.6^b
0 min + SM	89.1 ± 2.5^b	82.9 ± 5.2^{ab}	86.8 ± 4.9^b	91.8 ± 5.6^b
+ 2 h + SM	70.0 ± 6.9^a	64.9 ± 3.5^a	68.6 ± 4.4^{ab}	70.3 ± 0.6^{ab}
+ 24 h + SM	66.7 ± 4.3^a	-	70.6 ± 1.2^{ab}	-

Mean ± SE (n = 4)
Significance (P < 0.05) *a* - Control versus treatment; *b* - SM versus treatment
Biochemical and histological studies were carried out in mice administered with three injections of quercetin.

Table 3. Effect of 2 LD_{50} of percutaneously administered SM on MDA, GSH and GSSG of liver and protection by quercetin*

Group (First dose of quercetin)	MDA (%) Quercetin		GSH (%) Quercetin		GSSG (%) Quercetin		DNA frag. (%) Quercetin
	100 mg/kg	200 mg/kg	100 mg/kg	200 mg/kg	100 mg/kg	200 mg/kg	200 mg/kg
Control	99.9 ± 1.7	-	99.7 ± 2.6	-	99.2 ± 3.0	-	28.9 ± 0.2
Only SM	149.6 ± 8.5^a	-	46.1 ± 1.4^a	-	55.0 ± 0.3^a	-	47. ± 3.9^a
– 30 min + SM	97.0 ± 5.7^b	94.7 ± 3.4^b	62.6 ± 7.3^a	77.7 ± 6.6^{ab}	85.8 ± 7.9^b	91.6 ± 5.0^b	31.1 ± 3.2^b
0 min + SM	108.3 ± 2.5^b	100.7 ± 4.1^b	84.9 ± 8.8^b	101.6 ± 11^b	103.4 ± 9.4^b	94.8 ± 3.5^b	32.7 ± 1.6^b
+ 2 h + SM	124.2 ± 6.5^{ab}	104.2 ± 5.2^b	40.2 ± 3.8^a	53.5 ± 3.8^a	50.5 ± 5.2^a	55.8 ± 5.9^a	56.1 ± 5.8^a
+24 h + SM	115.0 ± 1.5^b	126.5 ± 13.6^b	35.5 ± 2.9^a	47.3 ± 2.3^a	45.2 ± 1.8^a	55.5 ± 6.5^a	-

Mean ± SE (*n* = 4)
MDA = 3.86 ± 0.10 n moles/g tissue; GSH = 3.50 ± 0.1 μ moles/g tissue; GSSG = 1.86 ± 0.33 μ moles/g tissue
Significance P < 0.05 *a* - Control versus treatment; *b* - SM versus treatment
*Quercetin 3 doses (i.p.).

Table 4. Effect of 2 LD_{50} of percutaneously administered SM on WBC, RBC and haemoglobin of blood, and protection by quercetin*

Group	WBC (%)		RBC (%)		HB (%)	
(First dose of quercetin)	100 mg/kg	200 mg/kg	100 mg/kg	200 mg/kg	100 mg/kg	200 mg/kg
Control	99.6 ± 9.7	-	102.9 ± 2.4	-	102.6 ± 3.3	-
Only SM	119.2 ± 15.3	-	130.9 ± 2.4^a	-	120.1 ± 8.6	-
– 30 min + SM	84.9 ± 11.5	119.2 ± 15.3	106.4 ± 2.2^b	103.5 ± 2.7^b	103.5 ± 2.8	99.9 ± 1.7
0 min + SM	109.4 ± 7.1	108.6 ± 12.3	106.1 ± 4.1^b	103.5 ± 3.9^b	103.7 ± 2.0	100.8 ± 2.7
+ 2 h + SM	93.3 ± 10.7	104.2 ± 5.2	126.4 ± 7.0^a	125.8 ± 4.0^a	129.6 ± 11.1^a	131.5 ± 11.3^a
+ 24 h + SM	92.4 ± 23.0	126.4 ± 13.6	126.4 ± 5.7^a	138.2 ± 4.0^a	124.9 ± 5.7	136.7 ± 6.1^a

Mean ± SE (*n* = 4)
WBC = 13.4 ± 0.7 x 10^3 cells/μl; RBC = 8.4 ± 0.4 x 10^6 cells/μl; Hb = 13.2 ± 0.4 g/dl
Significance P < 0.05 : *a* - Control versus treatment; *b* - SM versus treatment
*Quercetin 3 doses.

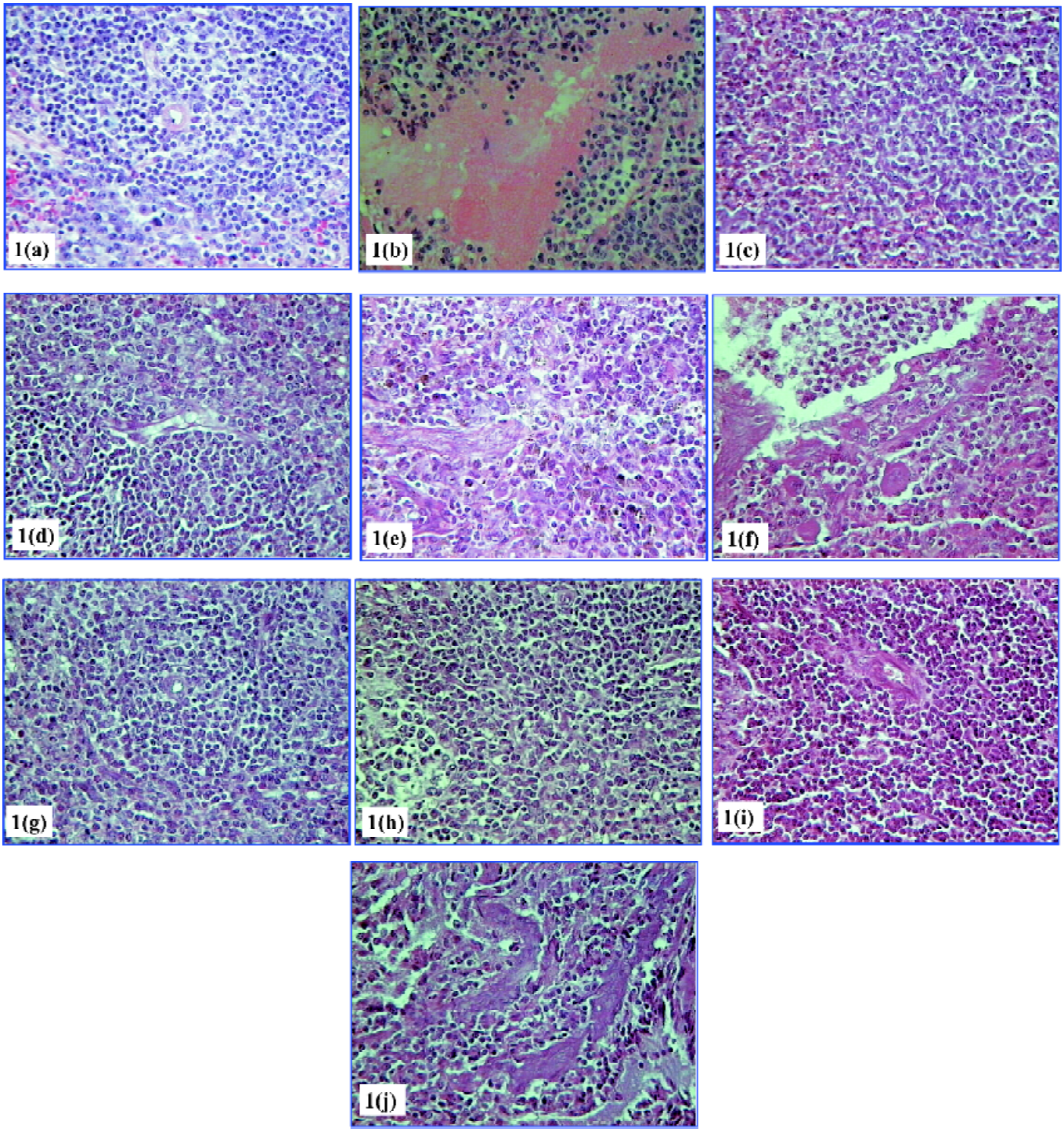

Figure 1. Photomicrographs of mice spleen; H and E, 100x: (a) control spleen showing normal histology of germinal centre, red and white pulp follicles, (b) SM-administered mice spleen (2 LD_{50}) showing congestion, hypocellularity and parenchymal atrophy, (c and d) pre- and simultaneous treatment respectively of 100 mg/kg of quercetin with SM, and (g and h) pre- and simultaneous treatment respectively of 200 mg/kg of quercetin with SM, showing near normal histology of spleen, (e and f) 2 h and 24 h post-treatment respectively of quercetin 100 mg/kg with SM, and (i and j) 2 h and 24 h post-treatment respectively of quercetin 200 mg/kg with SM, showing occasional haemorrhagic foci and necrotic red pulp follicles in the spleen.

Spleen histology of control mice showed normal structure having white pulp, red pulp, germinal centre and periarteriolar lymphatic sheath (PALS) [Fig. 1(a)]. Histological observation of spleen of mice, treated with SM showed severe congestion and hypocellularity of white pulp follicles. The spleen capsule was occasionally detached from the underlying parenchyma. This was partly caused by parenchymal atrophy and degenerative changes in the extracellular space leading to an overall shrinkage of the parenchyma and hypocellularity [Fig. 1(b)]. Quercetin at a dose of 100 mg/kg and 200 mg/kg doses reduced the severity of the lesions. Pre-treatment for 30 min and simultaneous treatment of quercetin showed near-normal histology [Figs 1(c), 1(d), 1(g) and 1(h)]. Occasional small foci of haemorrhage and necrosis remained in the red pulp of spleen in the 2 h and 24 h post-treatment groups of quercetin [Figs 1(e), 1(f), 1(i) and 1(j)]. Post-treatment for 24 h showed the least recovery, with similar but more severe pathologies than the post-treatment of quercetin for 2 h. A comprehensive account of various histopathological lesions in the splenic parenchyma and their protection by simultaneous treatment of quercetin is shown in Table 5.

Table 5. Splenic lesions induced by percutaneously administered SM at a dose of 2 LD_{50} and its protection by simultaneous treatment of quercetin

Lesion	SM	SM quercetin 100 mg/kg	SM quercetin 200 mg/kg
White pulp hypocellularity	+++	++	++
Atrophy of PALS	+++	+	+
Congestion and haemorrhage	+++	+	+
Activation of megakaryocytes	++	+	+
Condensation of chromatin	++++	++	+
Necrosis/Apoptosis	++++	++	++
Accumulation of fibrinoid material	+++	+ +	++

Grading : - nil, + minimal (< 12 %), ++ mild (< 22 %), +++ moderate (< 45 %) and ++++, severe (> 45 %).

The animals were sacrificed 7 days after SM administration. Quercetin was given as three doses with the first dose given simultaneously and two more doses on the subsequent days.

Histological features of control mice showed normal hepatic chord, hepatic lobules and hepatocytes [Fig. 2(a)]. Liver histology of mice after SM administration revealed hepatocellular cytoplasmic vacuolar changes characterised by granulovacuolar degeneration and perinuclear clumping of cytoplasm. Vacuolar degeneration of hepatocytes was predominantly observed in the intermediate zone with vacuolisation of centrilobular zone. The prominent lesions were cell swelling, cytoplasmic vacuole formation, and accumulation of inflammatory cells composed of lymphocytes and mononuclear cells. Sporadic neutrophils were also observed in the degenerated hepatic parenchyma [Fig. 2(b)]. The severity of the lesions was markedly reduced with quercetin administration at 30 min pre-treatment and simultaneous treatment at a dose of 100 mg/kg and 200 mg/kg. However, there was occasional vacuolar degeneration, nuclear pyknosis/necrosis and vascular congestion [Figs 2(c), 2(d), 2(g) and 2(h)]. The protective effect of 100 mg/kg and 200 mg/kg of quercetin given simultaneously with SM is shown in Table 6. Post-treatment for 2 h and 24 h of quercetin did not reduce the severity of the hepatic lesions [Figs 2(e), 2(f), 2(i) and 2(j)].

The histology of control mouse skin consisted of the three layers, viz., epidermis, dermis and hypodermis. The epidermis was thin and consisted of three layers dark stained outermost stratum cornium layer having flattened fused cells devoid of organelles, stratum granulosum comprising dense basophilic granular cells, and stratum spinosum

Table 6. Hepatic lesions induced by percutaneously administered SM at a dose of 2 LD_{50} and its protection by simultaneous treatment of quercetin

Lesions	SM	SM quercetin 100 mg/kg	SM quercetin 200 mg/kg
Disturbed lobular pattern	++	++	+
Haemorrhage	+++	+	++
Necrosis/apoptosis	+++	++	++
Obliteration of chromatin	+++	++	++
Proliferation of bile duct parenchyma	+++	+	++
Clumping of cytoplasm	++++	++	++
Hepatic vacuolation	++++	++	++
PMN. infiltration	++	-	+

Grading : - nil, + minimal (< 12 %), ++ mild (< 22 %), +++ moderate (< 45 %) and ++++, severe (> 45 %).

The animals were sacrificed seven days after SM administration Quercetin was given as three doses with the first dose given simultaneously and two more doses on the subsequent days.

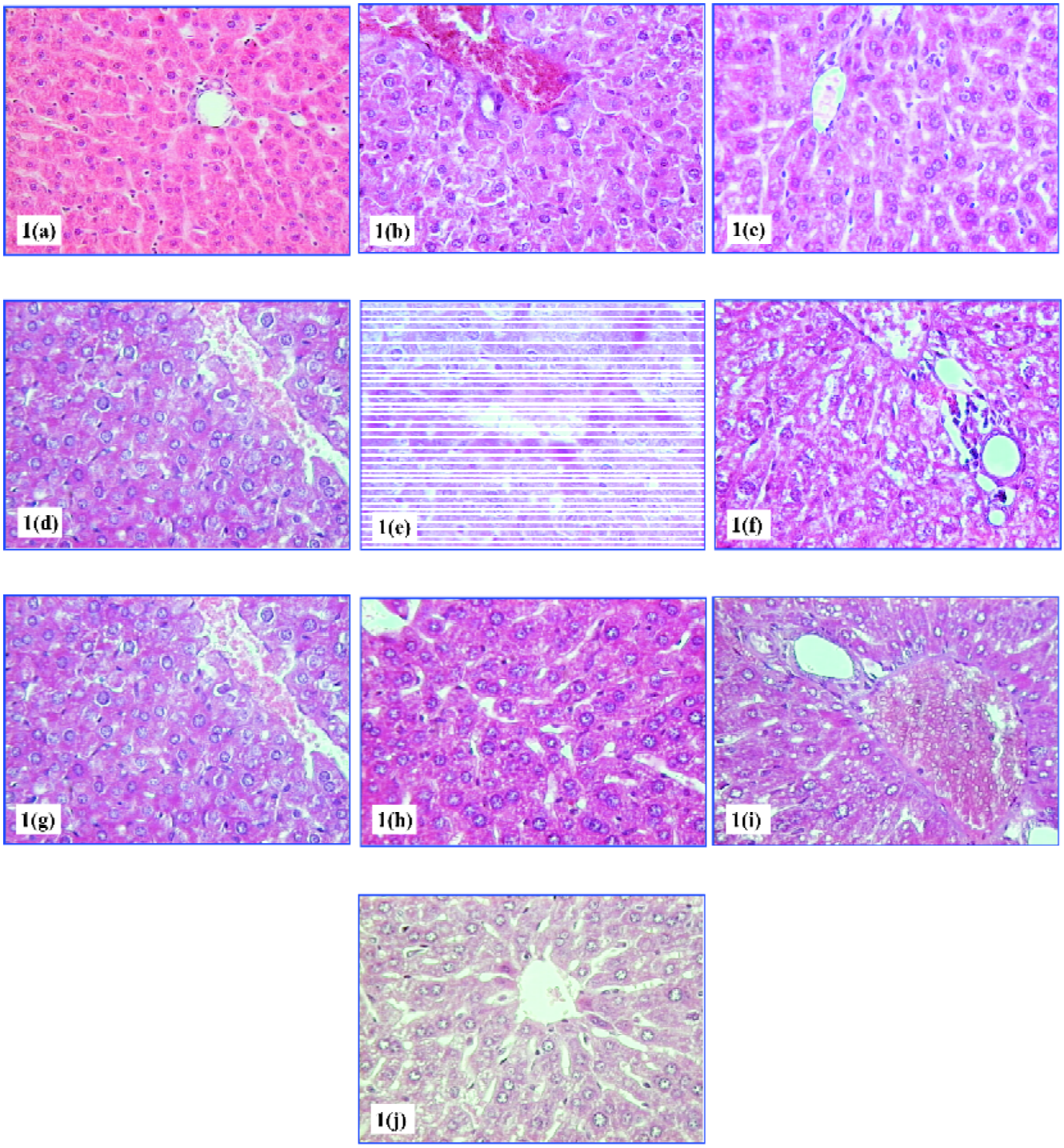

Figure 2. Photomicrographs of mice liver; H & E, 100x: (a) control liver showing normal hepatic parenchyma, hepatic lobules and hepatocytes, (b) SM-administered mice liver (2 LD_{50}) showing granulovacuolar degeneration and perinuclearing clumping of cytoplasm, (c and d) pre and simultaneous treatment respectively of 100 mg/kg of quercetin with SM, and (g and h) pre- and simultaneous treatment respectively of 200 mg/kg of quercetin with SM, showing reduced hepatic lesions, (e and f) 2 h and 24 h post-treatment respectively of quercetin 100 mg/kg with SM, and (i and j) 2h and 24 h post-treatment respectively of quercetin 200 mg/kg with SM, showing occasional hepatic parenchymal degeneration.

consisting of large polyhedral cells. The dermis contained loose connective tissue components located between epidermis and hypodermis [Fig. 3(a)]. Percutaneous application of SM in mice showed some degree of epidermal destruction as evidenced by focal keratinocyte swelling to extensive epidermal necrosis in stratum cornium layer. Swollen cells are seen throughout the epidermis. The spinous cells are enlarged and vacuolated while vacuoles are observed between epidermis and dermis. In some places, epidermis was completely sloughed off and dermis was completely exposed. Large amount of inflammatory infiltrate was also observed in the dermal region. The degenerated dermo-epidermal area was covered by exudates composed of fibrin, leucocytes, and RBC's. Intense inflammatory infiltrate in the dermis reached the hypodermis. SM administration also caused edema and ulcer [Fig. 3(b)]. Pre-treatment 30 min or simultaneous treatment of quercetin at doses of 100 mg/kg or 200 mg/kg with SM administration reduced the severity of the lesions [Figs 3(c), 3(d), 3(g) and 3(h)]. However, quercetin, 2 h or 24 h post-treatment following SM administration did not give significant protection to the skin lesions in comparison to pre- and simultaneous treatment of quercetin [Figs 3(e), 3(f), 3(i) and 3(j)].

4. DISCUSSION

Sulphur mustard is a potent alkylating agent with electrophilic property. This results in the production of reactive oxygen species (ROS) and oxidative stress with depletion of cellular detoxifying thiols including glutathione[33]. In this study, flavonoid quercetin was used which is a well known antioxidant, to reduce the toxic effects of SM[19]. A number of biochemical reactions in the body generate highly reactive oxygen species and free radicals with unpaired electrons that may cause cellular damage. An unpaired electron is highly unstable and reactive. Free radicals instantaneously react with other compounds to capture the needed electron. With the loss of an electron, the stable molecule per se becomes a free radical and a chain reaction is initiated[34]. Quercetin and its many metabolites can neutralise the free radicals by donating one of their electrons thus ending the electron chain reactions[35]. Quercetin is one of the most abundant flavonoids in human diet and is reported to exhibit a wide range of pharmacological properties[36]. In this study, intraperitonial administration of quercetin significantly prevented the SM-induced body weight loss and also protected the changes in different oxidative stress markers and hematological parameters.

Sulphur mustard is known to react with a wide variety of macromolecules[7]. The mortality depends upon the dose applied and the LD_{50} varies with the observation period[9]. Our earlier reported LD_{50} of SM by percutaneous route was 8.1 mg/kg and in this study the LD_{50} is estimated to be 9.7 mg/kg. Quercetin offered a dose dependent protection in mice. A dose of 100 mg/kg of quercetin gave a protection of two-fold compared to the protection of eight-fold at 200 mg/kg, when the first dose of quercetin was given simultaneously followed by two more doses on the subsequent days. Single dose of quercetin given simultaneously with SM gave a protection of 3.2-fold in 100 mg/kg and 5.6-fold in 200 mg/kg groups. In both the dose levels, 30 min pre-treatment as well as simultaneous treatments showed higher protection than post- treatments. The protection could be due to the direct interaction of quercetin or its metabolites with SM intermediates as well as its antioxidant property inside the system to nullify the effect of free radicals generated by SM.

The body weight of animals decreased progressively, and in some cases 50 per cent reduction in initial body weight was observed. This was partially due to the reduced intake of food and water as a result of the toxic effects of SM. Generally, the mice died within a period of 14 days after SM application and beyond this period they survived. At lower doses weight loss was seen up to 12 days after SM application, and then the body weight started improving. The protection on SM-induced weight loss was observed only when quercetin was administered as pre-treatment or simultaneous treatment but not as post- treatment. Similar observation was made in the protection of quercetin in ethanol-induced oxidative stress[20]. This shows that quercetin or its active metabolite should be available to interact with the active metabolite of SM. If this is true, than the two subsequent doses of quercetin given on the next two days may not be necessary. The

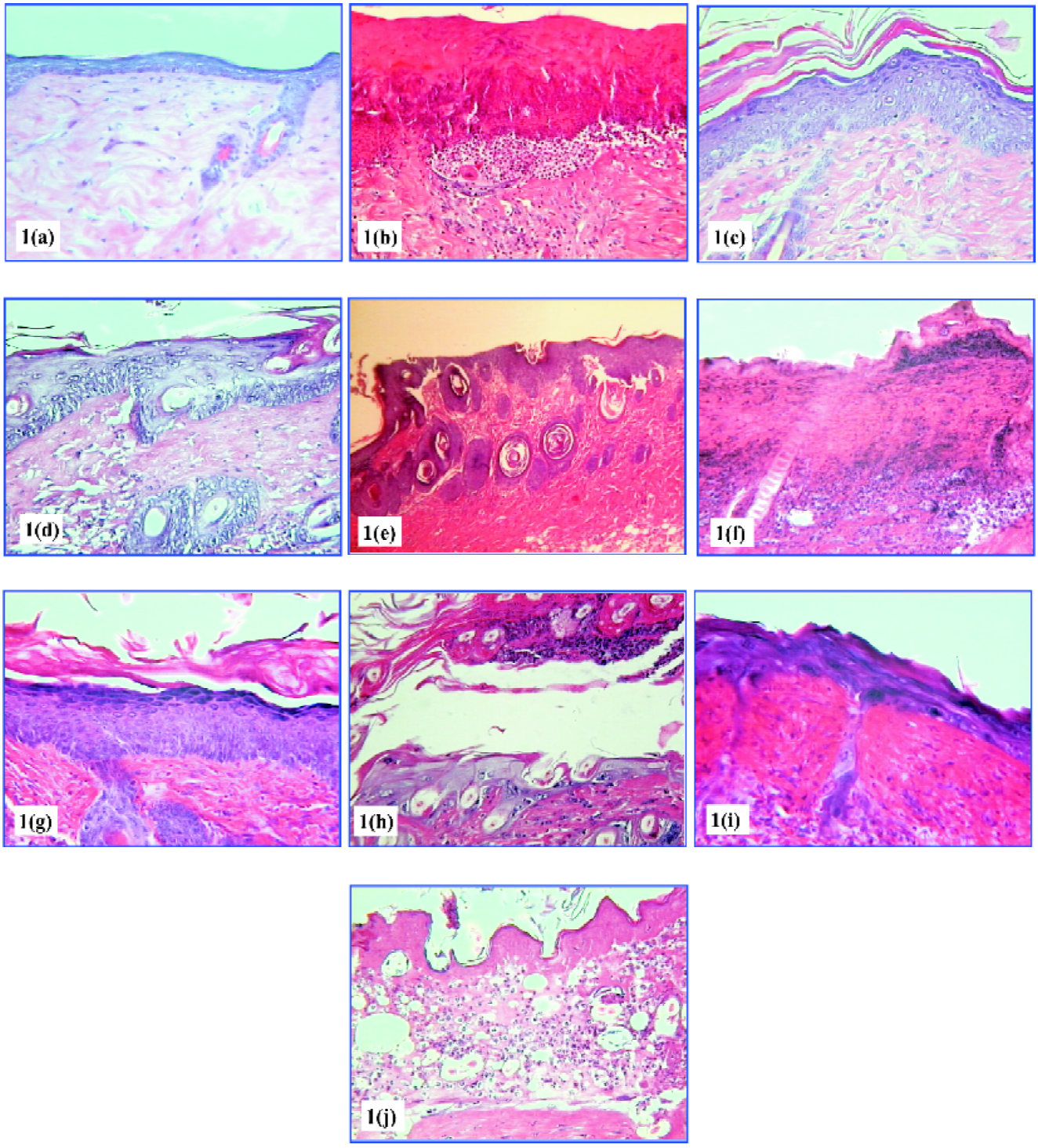

Figure 3. Photomicrographs of mice skin; H and E, 100x: (a) control skin showing normal epidermis and dermis having flattened fused cell, dense basophilic granular cells and large polyhedral cells with well defined cell borders; (b) SM-administered mice skin (2 LD_{50}) showing invasion of the epidermis by acute inflammatory cells, dermo-epidermal separation, accumulation of exudates composed of fibrinoid material and RBC's, (c and d) pre- and simultaneous treatment respectively of 100 mg/kg of quercetin with SM, and (g and h) pre- and simultaneous treatment respectively of 200 mg/kg of quercetin with SM, showing reduction of lesions in the skin; (e and f) 2 h and 24 h post-treatment respectively of quercetin 100 mg/kg with SM, and (i and j) 2 h and 24 h post-treatment respectively of quercetin 200 mg/kg with SM, showing lack of protection of SM-induced skin lesions.

initial experiments were planned with the three doses and later experiments were carried out with the single dose of quercetin given either as 30 min pre-treatment or simultaneous treatment. The protection of the single dose was at par with the three doses.

A significant decrease in GSH after SM administration is a constant finding. In the present study, a significant decrease not only in GSH but also in GSSG was observed. Since SM interacts with a wide variety of biomolecules causing multiorgan failure, decrease in GSH and GSSG is expected. The depletion of GSH may be due to direct interaction with sulphonium ion. The decrease in GSH and GSSG was recovered by quercetin in a dose-dependent manner. Pre-treatment and simultaneous treatment significantly protected but not the post-treatment. Quercetin's antioxidant properties are due to its active metabolites, which usually forms in the body by sulphation, methylation or glucuronation. The absorbed quercetin is transported through albumin, chiefly methylated in the 3' position and conjugated with glucuronic acid and sulphates. Recent studies have specified that the conjugated forms are mostly glucurono-sulphates. These active metabolites may be reacting with the intermediates of SM and free radicals to minimise the toxic effects. The post-treatments have failed because, quercetin metabolites should be present in the body before SM binds to the biomolecules.

Normally lipid peroxidation takes place in normal cells to some extant and due to SM toxicity, there is an increase in lipid peroxidation as shown by an increase in malondialdehyde (MDA) level. The MDA was increased in SM toxicity to 150 per cent and this was recovered by pre-treatment or simultaneous treatment with quercetin but not with post-treatment. Protection was more prominent in 200 mg/kg as compared to 100 mg/kg. This protection might be due to the quenching of lipid peroxides by quercetin from the cells. Quercetin also donates an electron to the free radicals formed in the body[20]. Since pre-treatment or simultaneous treatments alone could protect SM toxicity, it appears that the initiation of toxic effects following SM administration are immediate, though symptoms may appear later. SM is a bifunctional alkylating agent leading to DNA strand breaks and cell death[6]. The present study shows that pre-treatment and simultaneous treatment of quercetin, significantly protected SM-induced DNA damage. The authors expect that due to the antioxidant and free radical scavenging properties of the flavonoids, these might protect therapeutically. Hence, this study was carried out using various time points after application of SM and the results show that flavonoids will be beneficial only as a pre-treatment and not as post-treatment. SM forms sulphonium ion in the body which is electrophilic in nature. The flavonoids like quercetin are nucleophilic in nature and there is a possibility of an electron transfer from 3' *OH* group of quercetin to the sulphonium ion. This is one possible reason for the protection of quercetin as a prophylactic agent, by neutralising the sulphonium ion.

Following percutaneous administration of SM, the animals appear emaciated and dehydrated, resulting in reduced blood volume. This may be the reason of an increased hemoglobin and RBC count. SM causes bone marrow depression and a decrease in WBC count is expected. But no such decrease was observed, and WBC count was unaffected or slightly increased after SM. This is also due to the haemoconcentration. Another reason might be that bone marrow depression is expected at very high toxic dose of SM. Probably at the 2 LD_{50} dose, the effect of SM on haemopoitic cells is not that prominent for the depression of bone marrow. The present study also shows that percutaneous administration of SM can cause lesions distal to the site of application. Histological examination of spleen, liver, and skin showed that quercetin can protect SM-induced histological lesions and this may be due to its free radical scavenging properties.

Attention is now being focused on the flavonoids like quercetin which are pleiotropic in their effects, and over a dozen seemingly independent biological effects can be listed including its antioxidant property[37]. SM interaction with quercetin is one such interaction because of its neucleophilic characteristics. Quercetin is also a safe compound as the LD_{50} estimated in our establishment is more than 5.0 g/kg by the oral and i.p routes in mice. Further studies are in progress on various toxicological and biochemical properties of quercetin against SM toxicity.

ACKNOWLEDGEMENTS

The authors are grateful to Er. K. Sekhar, Director, DRDE, Gwalior, for his constant encouragement. Thanks are also due to Dr R.C. Malhotra and Mr K. Ganesan for providing sulphur mustard.

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