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ORIGINAL ARTICLE

Prophylactic efficacy of some chemoprotectants against abrin induced lethality

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ABSTRACT

Abrin is a highly toxic protein produced by *Abrus precatorius*. Exposure to abrin, either through accident or by act of terrorism, poses a significant risk to human health and safety. Abrin functions as a ribosome-inactivating protein by depurinating the 28S rRNA and inhibits protein synthesis. It is a potent toxin warfare agent. There are no antidotes available for abrin intoxication. Supportive care is the only option for treatment of abrin exposure. It is becoming increasingly important to develop countermeasures for abrin by developing pre- and post-exposure therapy. The aim of this study is to screen certain pharmaceutical compounds for their chemoprotective properties against abrin toxicity *in vivo* in BALB/c male mice. Twenty-one compounds having either antioxidant, anti-inflammatory and cyto-protective properties or combination of them, were screened and administered as 1h pre-treatment followed by exposure of lethal dose ($2\times LD_{50}$, intraperitoneally) of abrin. To assess the protective efficacy of the compounds, survival and body weight was monitored. Fifteen compounds extended the survival time of animals significantly, as compared to abrin. The following five of these compounds, namely: Epicatechin-3-gallate, Gallic Acid, Lipoic Acid, GSH and Indomethacin extended the life time ranging from 6 to 9 days. These compounds also attenuated the abrin induced inflammation and enzymes associated with liver function, but none of them could prevent abrin induced lethality. The compounds offering extension of life could be useful to provide a time-window for other supportive treatment and could also be used as combinatorial therapy with other medical countermeasures against abrin induced lethality.

KEY WORDS: Abrin; antidote screening; medical counter-measures; ribosome inactivating protein; ricin

Introduction

Abrin and ricin are potent phytotoxins belonging to the family of ribosome inactivating proteins (RIPs) that inhibit protein synthesis either directly by inactivating the ribsome or indirectly by modifying factors involved in translation of protein synthesis (Olsnes & Pihl, 1973).

Abrin shows significant similarities to ricin at the sequence and structure level, but abrin is several times more potent than ricin (Stirpe *et al.,* 1992). Abrin, like ricin, is currently considered a threat to public safety because of potential application in biological warfare or terrorist attacks (Olsnes *et al.,* 1978).

Both toxins are polypeptide toxins comprised of two dissimilar polypeptide chains, A chain and B chain held together by disulfide bond. The B chain is a galactose specific lectin and hence it binds to cell surface glycosylated receptors, which allows toxin entry, while the A chain having RNA N-glycosidase activity that irreversibly inactivates the 28S rRNA of the mammalian 60s ribosomal unit and arrests host cell protein synthesis (Endo *et al.,* 1987). In addition to its ability to inhibit protein synthesis, abrin is believed to adopt alternative mechanisms to trigger apoptosis. Inactivation of antioxidant proteins resulting in increased production of reactive oxygen species are also proposed to cause toxicity by abrin (Shih *et al.,* 2001). Abrin causes apoptosis in caspase dependent manner along with loss of mitochondrial membrane potential (Bora *et al.,* 2010). Ricin has been shown to induce lipid peroxidation, glutathione depletion and DNA damage in mice (Muldoon *et al.,* 1992). Abrin and ricin are also shown to induce localized and systemic inflammation (Dickers *et al.,* 2003; Griffiths, 2011). Currently, there is no FDA-approved therapeutics available for ricin and abrin exposure. Treatment is purely supportive and

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symptomatic. Thus the development of abrin countermeasures is urgent and important.

Since abrin has been shown to induce oxidative stress, inflammation, and cytotoxicity, we investigated the efficacy of a number of compounds with properties of inhibiting oxidative stress, inflammation or cytotoxicity. These included Celastrol, Sulforaphane, Galangin, Pinocembrin, Gossypin, N-acetyl Cysteine (NAC), Epicatechin-3-gallate (EGCG), Gallic Acid, Lipoic Acid, Ebselen, Naringin, Bay 11-7085, Amifostine, DRDE-07, Caffeic Acid, Melatonin, GSH, Quercetin, Prednisolon, Minocycline hydrochloride and Indomethacin (Saxena *et al*., 2014).

We administered a lethal dose of abrin which causes consistent lethality in mice. Using this condition with death as an end point, twenty one compounds or known drugs were screened against abrin toxicity. Fifteen compounds exhibited the ability to extend the survival time, of them five compounds extended the survival time up to or beyond 6 days. Though none of them prevented abrin induced death but at least these compounds were able to provide extension of life span up to a certain extent allowing to use other medical countermeasures.

Materials and methods

Chemicals

All kits for biochemical assessment were obtained from Erba Mannheim. Cytokine levels were estimated by using ELISA kit from R & D Systems. All other chemicals were obtained from Sigma Chemicals Co (St Luis, Missouri, USA), unless otherwise mentioned.

The following drugs were used for their potential as an antidote:

Celastrol, Sulforaphane, Galangin, Pinocembrin, Gossypin, NAC, EGCG, Gallic Acid, Lipoic Acid, Ebselen, Naringin, Bay 11-7085, Amifostine, DRDE-07, Caffeic Acid, Melatonin, GSH, Quercetin, Prednisolone, Minocycline hydochloride, Indomathacin. All compounds were obtained from Sigma-Chemical Co (St Luis, Missouri, USA) except DRDE-07. DRDE-07 is an amifostine analogue and synthesized in the Synthetic Chemistry Division of the Establishment.

Isolation of Abrin

Abrin was isolated from seeds of the white variety of *Abrus precatorius* using sepharose 6B affinity column chromatography and purified as described in a previous study (Kumar *et al.,* 2008). The purity and molecular weight of abrin protein was confirmed by coomassie blue staining and MALDI-TOF (data not shown). The stock protein solution was diluted with phosphate buffered saline (PBS, pH 7.4) to a concentration of 2 mg/ml.

Animals

Balb/c male mice randomly bred in the Institute's animal facility, weighing between 22–25 g were used in this study. The animals were housed in standard conditions of temperature and humidity. The animals were fed standard pellet diet (Ashirwad Brand, Chandigarh, India). Food and water were given *ad libitum*. The animals were handled according to the guidelines of the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA) and the Institutional Animal Ethics Committee (IAEC) approved the experiment with approval number Tox-57/55/NS.

Treatment regimen

Each treatment group consisted of six animals. The median lethal concentration (LD_{50}) of abrin with 95 percent confidence limits for intraperitoneal (ip) route was determined by the Gad and Weil method (Gad & Weil, 1989). For each dose (log dose) six mice were used and three to four doses were administered. After administration of abrin, the animals were observed for toxicity related symptoms and mortality till the 14th day post exposure. The LD_{50} of abrin with 95 percent confidence limit was calculated from table values and was found to be 1 µg/kg with confidence limit of 0.7–1.5 µg/kg. Only the abrin treated group was administered a single dose of 2×LD_{50} of abrin (2 µg/kg body weight respectively) by ip route. The compounds were tested as 1h pre-treatment followed by abrin exposure. The doses of the compounds and route of administration were chosen based on previous published literature and at least 3 doses were used to observe their efficacy. In cases where no previous published doses were available the dose was established based on preliminary study conducted at our lab. Those compounds which offered some protection were further repeated to confirm their protective efficacy at the minimum dose offering maximum protection. All compounds were administered either ip or intragastric (ig) (Table 1). Control animals received the same volume of vehicle control as the experimental group.

Assessment of efficacy of compounds

Screening of compounds was based on mean survival time. Change in body weight was also observed till the animal survived. All mice administered abrin 2×LD_{50} dose died within 2 days. Those antidotes which extended the life time beyond 5 days were further evaluated for other parameters. For further estimation of parameters, another set of groups was formed. In one group mice were treated with abrin 2×LD_{50}. Other groups were 1h pre-treated with compounds followed by abrin 2×LD_{50} exposure. Here we used the minimum dose of compounds offering maximum protection on the basis of survival time. All animals were anesthetized and euthenized on the 2nd, 4th and 6th day of treatment.

Determination of liver body weight index (LBI)

After sacrifice on day 2, 4 and 6 liver samples were quickly removed and washed to make free for adhering material, blotted and weighed to determine liver body weight index (LBI=liver weight ×100/body weight). The dissected liver was immediately frozen in liquid nitrogen and stored at –80 °C for further studies. In abrin only treatment groups all animals were sacrificed on day 2.

Table 1. Details of the compounds evaluated for protective efficacy against abrin toxicity.

S No	Compound	Doses used (mg/kg)	Solubility	Route of administration	Category	Property
1	Celastrol	2.5, 5, 10	Ethanol	ip	Triterpenoid	Antioxidant, anti-inflammatory
2	Sulforaphane	0.05, 0.5, 1, 10	DMSO	ip	Organosulfur compound	Antioxidant, anti-inflammatory
3	Galangin	1, 10, 20	DMSO	ig	Flavonoid	Antioxidant, anti-inflammatory
4	Pinocembrin	5, 20, 40	Ethanol	ig	Dihydroxyflavanone	Antioxidant, anti-inflammatory
5	Gossypin	10, 20, 30	Ethanol	ig	Pentahydroxyflavone glucoside	Antioxidant, anti-inflammatory
6	NAC	200, 400, 800 250, 500, 1000	Water	ig ip	Acetylated variant of L-cysteine	Antioxidant , free radical scavenger
7	EGCG	0.5, 2, 10 0.5, 2, 10	Water	ig ip	Bioflavonoids,	Antioxidant , free radical scavenger
8	Gallic Acid	50, 100, 150	Water	ig	Phenolic acid	Antioxidant ,free radical scavenger
9	Lipoic Acid	50, 100, 150	Ethanol	ig	Cyclic disulfide	Antioxidant , free radical scavenger
10	Ebselen	10, 50, 100	CHCl3	ip	Organo-selenium	Antioxidants , free radical scavenger , cytoprotectants
11	Naringin	1, 2	Ethanol	ip	Flavonoid	Anti-oxidant, anti-inflammatory ,anti-apoptotic
12	Bay 11-7085	1, 2.5, 5, 10	Ethanol	ip	Nitrite containing sulfonyl group	Anti-inflammatory, anti- apoptotic,
13	Amifostine	50, 100, 200	Water	ip	Organic thiophosphate prodrug	Antioxidants , Cytoprotectants,
14	DRDE-07	100, 200, 250	Water	ip	Amifostine analogue	Antioxidants ,anti-inflammatory Cytoprotectants,
15	Caffeic Acid	5, 10, 200 5, 10, 200	Ethanol	ip ig	Phenolic compound	Antioxidant, cytoprotectant
16	Melatonin	10, 20, 50	Ethanol	ip	Alkaloid	Antioxidant protects lipids, proteins, and DNA against oxidative damage.
17	GSH	50, 100, 200	Water	ig	γ-glutamylcysteinylglycine	Antioxidant, detoxification of xenobiotics
18	Quercetin	25, 50, 75	Ethanol	ig	Flavonoid	Antioxidant
19	Prednisolone	10, 15, 20, 25	Methanol	ip	Glucocorticoid corticosterone	Anti-inflammatory
20	Minocycline hydro-chloride	5, 25, 50	Water	ip	Tetracycline derivative	Anti-inflammatory
21	Indo-methacin	1, 5, 10	Ethanol	ip	Nonsteroidal anti-inflammatory drugs	Cyclooxygenase (COX) inhibitor

Liver lipid peroxidation assay

Measurement of malondialdehyde (MDA) was used as an index for lipid peroxidation in liver. It was carried out according to a previously described method of (Ohkawa *et al.,* 1979). The colorimetric reaction between MDA and TBARS was assayed (pH 2–3, 90 °C) for 15 min. The maximum absorption was recorded at 532 nm. The level of MDA was normalized with the total protein content.

Assessment of biochemical parameters

After the 2nd, 4th and 6th day of treatment blood was collected from retro-orbital plexus of mice before sacrifice. Serum harvested from each mouse at specified time points was used to determine serum activity of lactate dehydrogenase (LDH), alanine amino transferase (ALT) and aspartate aminotransferase (AST) and total bilirubin level by commercial diagnostic kits. In abrin only treatment groups all animals were sacrificed on day 2 and serum was stored.

Measurement of serum cytokines

Levels of inflammatory cytokines TNF-α, IFN-γ and IL-6 in serum samples were measured on day 2, 4 and 6 in compound treated group followed by abrin exposure, while on day 2 in abrin exposed group, using a standard sandwich ELISA according to the manufacturer's instructions.

Statistical analysis

Results are presented as mean±SEM. Values between control, toxin alone group and the antidote treated groups were compared using Student's t-test, with $p<0.05$ as the measure for significant differences.

Results

In the present study a number of compounds having antioxidant, anti-inflammatory, anti-apoptotic and cytoprotective properties or combinations of them were included. The details regarding solubility, dose administered and route of administration are given in Table 1. For each compound at least 3 doses were used. The LD_{50} of abrin in this study was calculated 1 µg/kg through ip route. Abrin at 2 µg/kg ($2\times LD_{50}$) consistently produced

Table 2. Protective efficacy of compounds against lethal dose of abrin in mice. Mice were treated with varying doses of compounds for 1h prior to abrin (2×LD_{50}) exposure.

S No	Compound	Dose and route of administration at which maximum protection offered (mg/kg)	Time to death (Days)
1	Control	NA	NA
2	Abrin (2LD_{50})	NA	1.9±0.50
3	Celastrol	All doses	2.2±0.62
4	Sulforaphane	0.5	4.0±0*
5	Galangin	10	2.6±0.50
6	Pinocembrin	All doses	2.0±0.50
7	Gossypin	20	4.1±0.80*
8	NAC	ig –all doses ip-250	2±0.7 3.6±0.25*
9	EGCG	ig - all doses - ip-2	2±0 7.5±1.5*
10	Gallic Acid	100	5.8±1.1*
11	Lipoic Acid	100	5.8±1.4*
12	Ebselen	All doses	2.4±0.5
13	Naringin	1	4.6±0.5*
14	Bay 11-7085	2.5	4.8±0.4*
15	Amifostine	50	3.3±0.5
16	DRDE-07	100	4.8±0.8*
17	Caffeic Acid	ig -all doses ip-all doses	2.60±.59 2.1±0.74
18	Melatonin	10	3±0.6*
19	GSH	50	6±1.1*
20	Quercetin	50	3.25±1.1
21	Prednisolone	20	3±0*
22	Minocycline hydrochloride	25	4±0.7*
23	Indomethacin	5	6.5±1.5*

Values are mean±SEM of six animals. *Significantly different from abrin group at $p<0.05$ by student's t test. The survival of mice was recorded daily and reported in days.

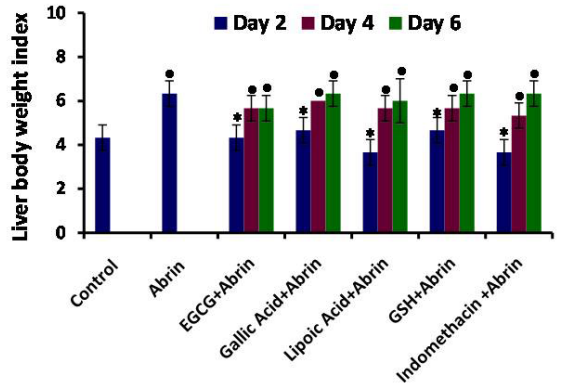

Figure 1. Protective potential of EGCG (2 mg/kg), Gallic Acid (100 mg/kg), Lipoic Acid (100 mg/kg), GSH (50 mg/kg) and Indomethacin (5 mg/kg) pre-treatment on liver body weight index after challenge with lethal dose (2×LD_{50}) of abrin on day 2, 4 and 6. Values are mean ± SEM of six animals in each group. * Significantly different from control for the same day and *significantly different from abrin at $p<0.05$ by student's t test.

100% lethality and the mean time to death varied from 1–2 days. Ip administration of abrin causes weight loss, drowsiness and diarrhea in mice. As mice were expected to die in about 1–2 days after abrin treatment, any significant delay in death by administration of this compound would be a protective effect. Table 2 summarizes the results on protective efficacy of the compounds. For each compound minimum dose offering maximum protection was calculated. A substantial number of compounds increased survival time but none of them protected from abrin induced lethality. Sulforaphane, Gossypin, NAC, EGCG, Gallic Acid, Lipoic Acid extended the survival time, but offered no protection from abrin induced lethality. Similarly Naringin, Bay 11-7085, Amifostine, DRDE-07, Melatonin, GSH, Predniosolone, Minocycline hydrochloride, Indomethacin extended the mouse survival but could not prevent lethality. Compounds having antioxidant, anti-inflammatory, antiapoptotic properties admistered alone or combinations of them provided extension of survival time suggesting no specific properties of compound responsible for protection. Among all the compounds which offered partial protection, five compounds EGCG, Gallic Acid, Lipoic Acid, GSH, and Indomethacin extended the survival time beyond 5 days. EGCG (2 mg/kg) by ip route was found to be the most potent antidote increasing the life time up to 7 days. Interestingly, EGCG did not provide any protection by ig route of administration. No toxicity was observed with any of the solvents or compounds when administered alone (data not shown). In the abrin treated group there was a drastic decrease in body weight on the second day, as compared to the initial weight, and all animals died on the 2nd day. On day 2 EGCG, Gallic Acid, Lipoic Acid, Naringin, Bay 11-7085, DRDE-07, Malatonin, GSH, Quercetin, Prednisolone, Minocycline, Indomethacin significantly protected against the body weight loss as compared to the decrease in weight in the abrin treated group. On day 4 and 6 there was further decrease in body weight in EGCG, Gallic Acid, Lipoic Acid, GSH, Indomethacin treated mice as compared to day 0, leading eventually to death (Table 3).

Effect of the compounds on LBI

Figure 1 shows the effect of pharmaceutical compounds on LBI. On day 2 there was significant increase in LBI in abrin treated animals as compared to control mice, but LBI in the groups treated with compounds were comparable to control. On day 4 and 6, LBI was found to be significantly increased in the group treated with compounds followed by abrin exposure as compared to control mice but still comparable to the abrin exposed group.

Effect of abrin and pre-treatment of compounds on liver lipid peroxidation

The deleterious effect of reactive oxygen species is measured by the amount of lipid peroxidation. MDA is commonly measured as a lipid peroxidation marker. There was more than a 3-fold increase in MDA formation in the abrin treated group compared to control.

Table 3. Effect of abrin and pre-treatment of compound against abrin toxicity on mice body weight.

Group	Day 0 Body weight(g)	Day 2 Body weight(g)	Day 4 Body weight(g)	Day 6 Body weight(g)
Control	23±0.5	24±0.8	26±0.8•	27±0.5•
Abrin(2×LD_{50})	24±0.5	19.6±0.3•	–	–
Sulforaphane(0.5 mg/kg)+Abrin(2×LD_{50})	23±0.9	21±0.7•	20±0.7•	–
Gossypin(20 mg/kg)+Abrin(2×LD_{50})	25±0.4	21±0.2•	18±0.5•	–
NAC(250 mg/kg)+Abrin(2×LD_{50})	23±1.0	18±0.4•	–	–
EGCG(2 mg/kg)+Abrin(2×LD_{50})	23±0.5	24±0.8*	23±0.64*	22±1.1
Gallic Acid (100mg/kg)+Abrin(2×LD_{50})	25±0.5	24±1.2*	23.5±0.3•*	21±0.6•
Lipoic Acid(100mg/kg)+Abrin(2×LD_{50})	25±0.2	25±1.2*	22±0.5*	20±0.3•
Naringin(1mg/kg)+Abrin(2×LD_{50})	24±1.5	23±1*	22±0.3•*	–
Bay 11-7085(2.5mg/kg)+Abrin(2×LD_{50})	25±0.4	23±0.8*	21±0.8•	–
Amifostine(50mg/kg)+Abrin(2×LD_{50})	24±0.7	19.7±1.5•	16±0.9•	–
DRDE-07(100mg/kg)+Abrin(2×LD_{50})	23±0.8	24±0.1*	23±0.35*	–
Melatonin(10mg/kg)+Abrin(2×LD_{50})	24±0.6	24.5±0.2*	20±1.2•	–
GSH(50mg/kg)+Abrin(2×LD_{50})	24±0.9	25±0.9*	23±0.5*	22±0.05•*
Quercetin(50mg/kg)+Abrin(2×LD_{50})	25±0.2	24±0.8*	22±0.1•*	–
Prednisolone(20mg/kg)+Abrin(2×LD_{50})	24±0.7	21±0.8*	–	–
Indomethacin (5mg/kg)+Abrin(2×LD_{50})	25±0.2	25±0.7*	23±0.9•*	20±1•

Significance $p<0.05$; *Abrin group (day 2) *vs.* treatment group (day 2, 4 and 6); • within same group day 0 *vs.* day 2, 4 and 6. The body weight of mice was recorded daily till the animal survived.

Pre-treatment of EGCG, Gallic Acid, Lipoic Acid, GSH caused significant reduction in lipid peroxidation as compared to the abrin treated group, but still their level was significantly higher than untreated mice at all three time points, suggesting partial protection offered by these compounds. Indomethacin pre-treatment was not able to suppress abrin induced MDA level at any time point studied (Figure 2).

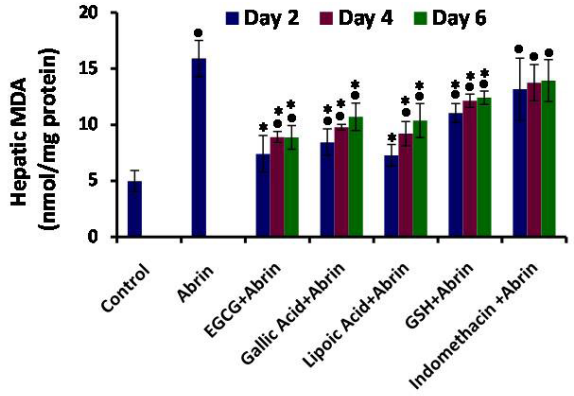

Figure 2. Protective effect of EGCG (2 mg/kg), Gallic Acid (100 mg/kg), Lipoic Acid (100 mg/kg), GSH (50 mg/kg) and Indomethacin (5 mg/kg) pre-treatment on hepatic MDA level after exposure to lethal dose (2×LD_{50}) of abrin on day 2, 4 and 6. Values are mean ± SEM of six animals each group. • Significantly different from control and *significantly different from abrin at $p<0.05$ by Student's t-test.

Effect of compounds on biochemical parameters

Serum enzymes AST, ALT, total bilirubin, LDH are the enzymes commonly used for liver cell integrity. Abrin exhibited toxicity as indicated by the significant increase in the level of these enzymes as compared to control. Pre-treatment of EGCG significantly brought down the level of serum AST, ALT, total bilirubin and LDH augmented by abrin but could not suppress them to the level comparable to control. Similar observation was found with gallic acid pre-treatment for serum AST, ALT and total bilirubin levels, except serum LDH. Serum LDH was found be the same as in abrin treated mice in Gallic Acid treated group on day 4 and day 6. Lipoic Acid pre-treatment also significantly attenuated serum AST, serum ALT, total bilirubin and serum LDH level as compared to levels in abrin treated mice. GSH pre-treatment also decreased the level of serum AST, ALT, total bilirubin and serum LDH activity increased by abrin. GSH and indomethacin pre-treatment could not inhibit serum AST activity on day 4 and 6, as compared to abrin. Indomethcin significantly decreased the total bilirubin level increased by abrin on all three days of the study. At the initial time point serum LDH and ALT were decreased by indomethacin pre-treatment but at a later time point there was further increase in the level, reaching the level of abrin treated mice (Figure 3).

Effect of compounds on pro-inflammatory cytokines

Serum IFN-γ, IL-6 and TNF-α are the cytokines associated with inflammation. We also examined the effect of pretreatment with these compounds on inflammatory cytokines. Abrin elevated the serum IFN-γ (339±46 pg/ml; 4 fold), IL-6 (364±60 pg/ml; 4 fold) and

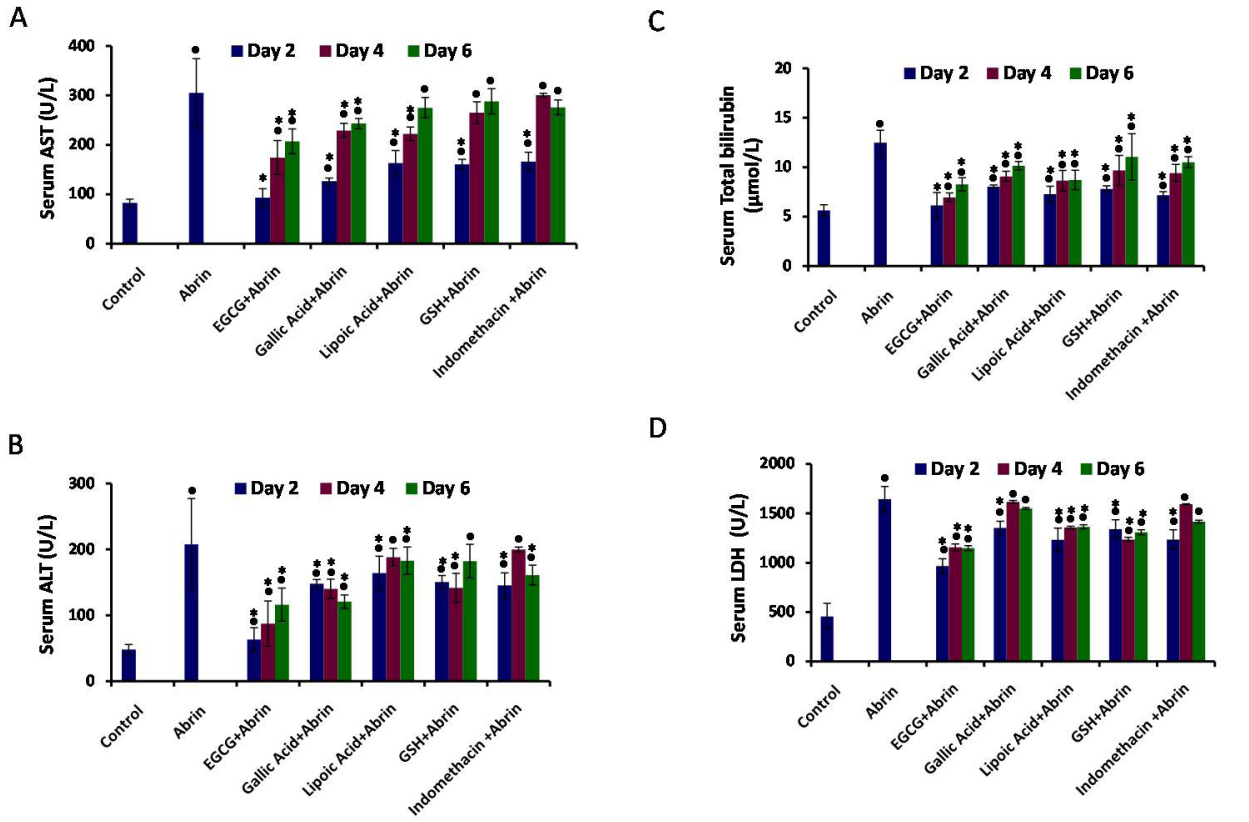

Figure 3. Protective efficacy of EGCG (2 mg/kg), Gallic Acid (100 mg/kg), Lipoic Acid(100 mg/kg), GSH (50 mg/kg) and Indomethacin (5 mg/kg) pre-treatment on (A) serum AST; (B) serum ALT; (C) serum total bilirubin and (D) serum LDH after challenge with lethal dose ($2\times LD_{50}$) of abrin on day 2, 4 and 6. Values are mean ± SEM of six animals each group. * Significantly different from control and *significantly different from abrin at $p<0.05$ by Student's t-test.

TNF-α (446±60 pg/ml; 5 fold) level as compared to control. All the compounds significantly decreased the level of all three cytokines as compared to abrin. Although gallic acid and lipoic acid pre-treatment on day 4 and 6 could not suppress TNF-α levels increased by abrin exposure.

Discussion

Abrin and ricin are most dangerous plant toxins and were classified as potential agents for biological warfare and bioterrorism by the Biological and Toxin Weapon Convention (BTWC, 2001). Though the mechanisms of toxicity of RIPs at cellular and molecular levels have been delineated previously, the development of antidote has proven elusive (Miller *et al.,* 2002). As one of the potential bioweapons, development of abrin countermeasure has received considerable attention. The immediate consequence of abrin poisoning is oxidative stress, inflammation, cytotoxicity. The mode of action at cellular level is the specific inhibition of protein synthesis. Studies aimed at finding an antidote for ricin have shown significant extension of survival time in mice (Muldoon & Stohs, 1994). Earlier studies also indicate a possible role for free radical scavenger in antagonizing abrin induced toxicity (Saxena *et al.,* 2014).

Few antioxidants, anti-inflammatory and cyto-protective compounds have been shown to counteract the oxidative damage and inflammation produced by toxicants (Muldoon & Stohs, 1994; Sorrenti *et al.,* 2013). The compounds of these categories were therfore screened for their ability to inhibit abrin induced toxicity by reducing oxidative stress and inflammation. Of the compounds tested, a few provided significant extension of survival time, while the other compounds had no effect at the doses used in the study.

Compared to abrin treatment where all animals were died within 2 days, 15 compounds extended the survival time ranging from 3 days to 7 days but their body weight decreased drastically. EGCG, Gallic acid, Lipoic Acid, GSH and Indomethacin increased protection beyond 5 days and maximally up to 7 days. All these 5 compounds suppressed the oxidative stress, inflammation and liver function associated parameters elevated by abrin exposure, but none of them could induce decrease up to the level of control animals. Among all the compounds studied, maximum life time extension was provided by EGCG. EGCG is the major catechin found in green tea and functions as a powerful antioxidant, preventing

oxidative damage in healthy cells. In the present report EGCG effectively decreased the abrin induced level of MDA, inflammatory cytokines, oxidative stress, serum AST, ALT, total bilirubin and serum LDH as compared to gallic acid, lipoic acid, GSH and indomethacin pretreatment. In our previous study, abrin has been shown to induce Fas pathway of apoptosis, It could be possible that EGCG suppresses abrin toxicity by inhibiting expression of the ligand of death receptor (Fas L), as shown in cisplatin induced nephrotoxicity. EGCG increases the activity of phase II detoxifying enzymes in mouse liver and blocks a wide array of signal transduction pathways, which may be another reason of highest protection achieved by EGCG. Protection by EGCG by ip route not by ig route suggested that the route of administration greatly matters and affects bioavailability. In concordance with previous reports it is suggested that ig administration of EGCG is not effective due to inefficient absorption or metabolism in the digestive tract (Yuan *et al.,* 2012). A similar observation was found with NAC where *ig* administration was not effective, while *ip* administration marginally increased the survival time (Saxena *et al.,* 2013; Zou *et al.,* 2014; Singh *et al.,* 2011). Via ig and oral route of administration, NAC undergoes deacetylation and produces cysteine, a precursor of glutathione. We thought it appropriate to present glutathione (Shalansky *et al.,* 2005) as this route may be beneficial to suppress abrin induced oxidative stress. But unfortunately, no protection was observed using the ig route of administration. Since abrin has also been shown to induce hepatotoxicity (Niyogi, 1977), we hypothesize that NAC via ip route may reach the liver and neutralizes the toxicity. Although the ip route of NAC has offered protection by extending the life span of mice against abrin toxicity but it was less significant as compared to EGCG, Gallic acid, Lipoic acid, GSH and Indomethacin. In the cisplatin induced nephrotoxicity model of the rat, NAC was tested by ip, oral, intravenous (iv) and intra-artrial (ia) route. There was no protection with oral and ip route but the iv and ia route of administration showed significant protection, suggesting that the route of administration can have a profound effect on the efficacy of chemoprotectants and an elaborated study is warranted with using the iv and ia route of administration against abrin toxicity (Dickey DT *et al.,* 2008).

In the present report, abrin is shown to deplete glutathione level and increase lipid peroxidation, similarly to previous studies where ricin treatment was shown to elevate lipid peroxidation (MDA), while GSH was decreased in both liver and kidney (Kumar *et al.,* 2003, Muldoon *et al.,* 1992). Keeping in mind the ability of GSH to replenish glutathione level and attenuate lipid per oxidation, it was tested for its ability to reduce abrin toxicity. GSH increased the life span up to 6 days. Surprisingly, NAC and amifostine, possessing a similar property of thiol modulation, were not able to protect the mice up to the same extent. DRDE-07, which is an amifostine analogue, significantly extended the survival time and partially better than amifostine, which may be due to the presence of an aryl group in DRDE-07 which increases its lipophilicity and thus its bioavailability (Kerksick & Willoughby, 2005; Vijayaraghavan *et al.*, 2001). Gallic acid and lipoic acid, well known antioxidants and free radical scavengers, extended the survival time up to 6 days, while Galangin, Pinocembrin, Ebselen Caffeic Acid, which are also having antioxidant property, did not offer any protection. Flavonoids are another group of cytoprotectans which donate the hydroxyl group to the free radicals, sparing GSH to interact with other free radicals. Naringin, belonging to the group of flavonoids occurring naturally in citrus fruit, extended the survival time up to 4 days, while quercetin, which is also a flavonoid, did not offer the same protection. Similarly to Naringin, Gossypin is

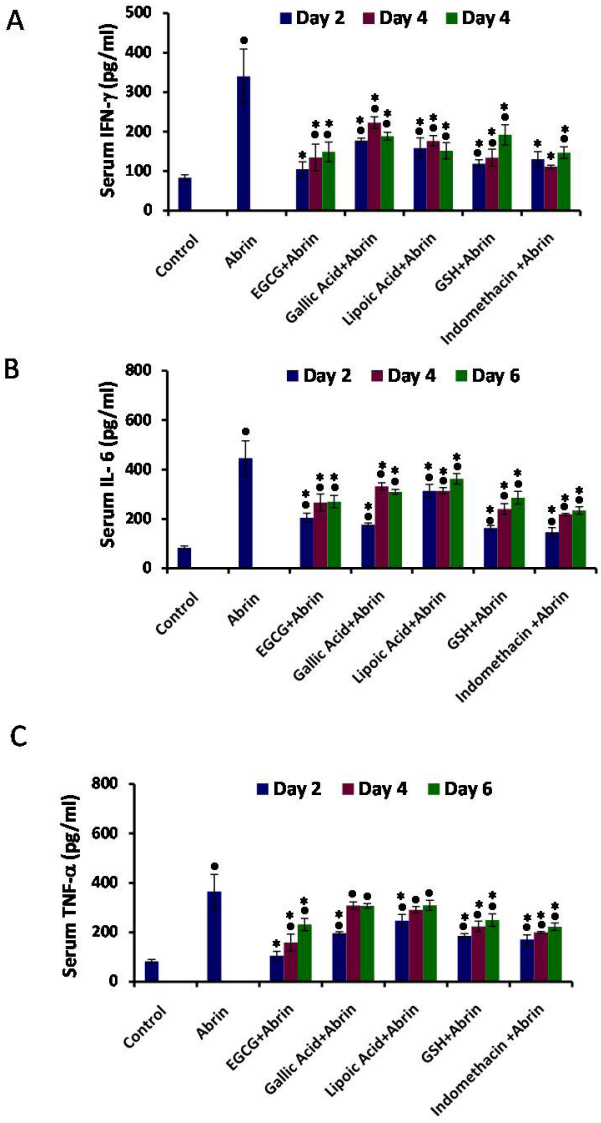

Figure 4. Protective potential of EGCG (2 mg/kg), Gallic Acid (100 mg/kg), Lipoic Acid (100 mg/kg), GSH (50 mg/kg) and Indomethacin (5 mg/kg) pre-treatment on inflammatory cytokine levels after exposure to lethal dose (2×LD_{50}) of abrin on day 2, 4 and 6. (A) serum IFN-γ level; (B) serum IL-6 level; (C) serum TNF-α level. Values are mean ± SEM of six animals each group. * Significantly different from control and *significantly different from abrin at $p<0.05$ by Student's t-test.

another flavonoid. It exhibited anti-inflammatory action and increased the life span up to 4 days. Indomethacin is a known non-steroidal anti-inflammatory drug which could extend the life span significantly, possibly by suppressing abrin induced inflammation. Bay11-7085 and prednisolone are further anti-inflammatory compounds which offered significant increase in life span but less than did indomathacin (Strickson *et al.,* 2013; Garg *et al.,* 1994).

Suforaphane, Melatonin and Minocycline also provide significant extension of survival time owing to antioxidant and anti-inflammatory activity, while celastrol did not provide any protection in spite of having similar properties.

A few compounds of antioxidant and anti-inflammatory activity are offering protection, while other compounds having the same property are not. This is suggesting that differences in chemistry between these compounds may be responsible for different efficacy. Another reason could be that the structural difference in the compounds leads to different mechanisms of action for protection or difference in bioavailability at the site of action. Further modulation of abrin toxicity may also depend upon pharmacokinetics, bioavailability and doses of compounds. Those compounds offering protection were also tried for repeated treatment but could not provide any additional significant protection (data not shown). In the present study, one of the limitations is lack of data of compound efficacy after abrin treatment. But no beneficial effects of repeated treatment of few compounds suggest these compounds may be not beneficial for therapeutic treatment. Since these compounds are offering extension of life, combination of these treatments among themselves as well with other treatment modalities may be useful to inhibit abrin toxicity.

Only life time extension for a few days and then death suggests at a later course of action of abrin toxicity some other pathways to be dominating, which are responsible for the death of mice. Abrin toxicity is shown to associate with multiple modes of cell death, as inhibition of protein synthesis leads to activation of MAPK pathway and caspase 3 activation. Abrin is also shown to exert cytotoxicity via other pathways like receptor mediated extrinsic pathway as a secondary consequence of toxicity. Further cross talk between these pathways may aggravate the toxicity. It can be concluded that our compounds are only neutralizing the consequences of toxin up to some extent but increase in all parameters associated with stress at later time points and death after some time suggest that direct neutralization of toxin alone or combination with these compounds may be ideal approach and good therapeutic option.

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