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Anticlastogenic Effect of Redistilled Cow's Urine Distillate in Human Peripheral Lymphocytes Challenged With Manganese Dioxide and Hexavalent Chromium

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Objective To study the anticlastogenic effect of redistilled cow's urine distillate (RCUD) in human peripheral lymphocytes (HLC) challenged with manganese dioxide and hexavalent chromium. **Methods** The anticlastogenic activity of redistilled cow's urine distillate was studied in human polymorphonuclear leukocytes (HPNLs) and human peripheral lymphocytes *in vitro* challenged with manganese dioxide and hexavalent chromium as established genotoxicants and clastogens which could cause induction of DNA strand break, chromosomal aberration and micronucleus. Three different levels of RCUD: 1 μL/mL, 50 μL/mL and 100 μL/mL, were used in the study. **Results** Manganese dioxide and hexavalent chromium caused statistically significant DNA strand break, chromosomal aberration and micronucleus formation, which could be protected by redistilled cow's urine distillate. **Conclusion** The redistilled cow's urine distillate posseses strong antigenotoxic and anticlastogenic properties against HPNLs and HLC treated with Cr^{+6} and MnO_2. This property is mainly due to the antioxidants present in RCUD.

Key words: Redistilled cow's urine distillate (RCUD); DNA strand break; Clastogenicity; Chromosomal aberration; Micronuclei; Hexavalent chromium and manganese dioxide

INTRODUCTION

The revered Indian cow, known as 'Kamdhenu' in Indian scripts, is believed to be a "mobile hospital", for most of the diseases. A number of incurable diseases can be cured by regular use of medicines derived from cow.

Urine of cow is elaborately described in ancient scriptures like *Charak-Sanhita*, *Rajnighantu*, *Brahad-Wagbhatt*, *Shshrut Sanhita* and *Amritsagar*, as bitter, pungent, piquant, spicy, warm and full of all the five types of elixirs. It is an anti-poisonous insecticide and a regulator for disorders like gas, acidity and cough. It promotes power of wisdom in human beings, acts like a universal medicine and is easily digested by all[1-2].

The root cause of various diseases in human beings is believed to be due to shortage or accumulation of certain elements, which are already in the body. The urine of cow contains all such elements. Hence, according to Ayurveda, it is considered as a natural and universal medicine to fulfill the shortage of element or to equalize and reduce the increased elements level in the body by restoring the excretion mechanisms of the body. For patients with cancer, the urine of cow and essence of dung appear to be the alternative to chemotherapy, and have no side effects[3-4]. Though Indian Ayurvedic literature cites about the medicinal properties of cow urine, there is very little scientific evidence that supports the literature. Recently scientific attempts have been made to support the view[5].

The genotoxic effect of Cr^{+6} is cited in many literatures. Treatment of human lymphocytes with Cr^{+6} results in a significant increase in the number of micronuclei and an induction of sister chromatid exchange through production of reactive oxygen species[6]. Manganese dioxide has also been known to cause genotoxicity/clastogenicity through production of ROS[7]. These oxygen species, if not scavenged can cause oxidative damage to DNA, as well as to precursors of DNA (such as GTP), resulting in

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mutation. Such mutations have been implicated in a number of human diseases.

In the present investigation, a study was carried out along with short term genotoxicity studies to assess the antigenotoxic and anticlastogenic potential of redistilled cow's urine distillate (RCUD) against MnO_2- and Cr^{+6}-induced genotoxicity and clastogenicity in human polymorphonuclear leukocytes and lymphocytes. Pretreated and simultaneously treated (RCUD) cells were exposed to Cr^{+6} and MnO_2.

MATERIALS AND METHODS

Chemicals and Media

Dulbacco's modified eagles media (DMEM) for culturing human lymphocytes, cytochalasin-B (Cyt-B), colchicine and dimethyl sulfoxide (DMSO) was purchased from Sigma, (St. Louis, MO, USA). Ethidium bromide, sodium sulphate and D-glucose were procured from Hi-Media Laboratories, India. Tris-HCl, ammonium chloride, potassium dichromate, trypan blue, sodium hydroxide and potassium chlorides were obtained from Sisco Research Laboratories, India. Penicillin, streptomycin and phytohemagglutinin M were purchased from GIBCO, Invitrogen Corporation (UK). Heat-inactivated fetal bovine serum was purchased from Life Technology (UK), while Giemsa stain was obtained from BDH (Santa Monica, CA).

Distillation of Cow Urine

Cow urine collected freshly from the local cowshed, was distilled at 100℃ using a temperature-controlled distillation apparatus. The single distilled cow urine was acidified by lowering the pH below 2.0 with the addition of 85% orthophosphoric acid. The cow urine was again distilled at 100℃ using a temperature controlled distillation apparatus to remove ammonia present in the distillate. The redistilled cow urine distillate (RCUD), with volatile acid content ranging between 1100 and 1300 mg/L, was used for the ameliorative study.

Fluorometric Analysis of DNA Unwinding Assay

The human polymorphonuclear leukocytes (HPNLs), 5×10^6 cells/mL were treated with a test solution having the final concentration of Cr^{+6} (600 µmol/L) and MnO_2 (1.2 mmol/L), different concentrations of RCUD (1 µL/mL, 50 µL/mL and 100 µL/mL), 0.1% DMSO and sterile distilled water (negative control) in a final volume of 1 mL, for 1 hour. To study the ameliorative effect of the RCUD on DNA strand breaks induced by test chemicals, the experiment was divided into two sets. In the first set, HPNLs were pre-incubated with RCUD for 1 hour prior to addition of Cr^{+6} and MnO_2 (pre-treatment). In the second set, the cells were treated with Cr^{+6} and MnO_2 along with RCUD simultaneously, and were further incubated for 1 hour in a 5% CO_2 incubator at 37℃. The treatment was terminated by additing of 4-5 mL ice-cold saline (0.9 % NaCl). The treated and control cells were centrifuged at 400× g for 10 minutes at 4℃, pellet was obtained and resuspended in solution B and the volume was made up to 2.0 mL. The suspended HPNLs were processed for FADU assay as described elsewhere[8-9].

Clastogenic Assay

About 0.5 mL of human venous blood was added to 3.5 mL of DMEM (human leukocyte culture media) supplemented with 20% of fetal bovine serum to which phytohemagglutinin (PHA) (50 g/mL), antibiotics (Penicillin 100 IU/mL and Streptomycin 50 µg/mL) and heparin sodium salt (5000 IU) (0.4 mL/100 mL) was added and incubated at 37℃ for 72 h depending on the experimental conditions. Twenty-four hours after culture initiation, human peripheral lymphocytes (HLCs) were treated with RCUD at different concentrations (100 µL/mL and 200 µL/mL) and clastogens MnO_2 (1.2 mmol/L) and Cr^{+6} (5 µmol/L) to study their genotoxic effects. For modulatory effect, the cells were pretreated and simultaneously treated with RCUD and test chemicals. The experiment was divided in two sets. In the first set, cells were pre-incubated with RCUD for 1 hour prior to addition of clastogens Cr^{+6} and MnO_2, further incubated for 3 hours, whereas in the second set, lymphocyte cultures were treated simultaneously with RCUD, MnO_2 and Cr^{+6} for 3 h.

Chromosomal Aberration Assay

After treatment, the cultures were washed, refed with a complete medium and further incubated in a 5% CO_2 incubator at 37℃ for 72 h. The cultures were treated with colchicine (0.1 µg/mL) 2 h before harvesting and further processed for preparation of slides for chromosomal aberrations[10]. One hundred-well spread metaphases were scored for aberration study, namely chromatid and chromosome breaks, fragments, exchanges, rings, gaps.

Micronucleus Assay

After treatment, the cultures were washed, refed with a complete medium and further incubated in a 5% CO_2 incubator at 37℃ for 72 h. At 44 h, the cultures were treated with 0.6 µg/mL of Cytochalasin

B to arrest the cells in a binucleated state and incubated till the completion of assay (72 h). At the end of the incubation period, the cultures were processed and the slides were stained with 4% Giemsa stain for 10 min. About 2000 binucleated cells with well-preserved cytoplasm were scored for the presence of micronuclei[11].

Statistical Analysis

The mean $\bar{x} \pm s$ was calculated for each parameter. The data were analyzed using one-way ANOVA test. The results were compared with control samples in order to assess the statistically significant genotoxicity and clastogenicity induced by chemical treatment and the effect of RCUD treatment. To evaluate the protective property of the distillate, the pretreated and simultaneously treated cultures (RCUD + clastogens) were compared with the cells exposed to clastogens alone.

RESULTS

The pH of the distilled cow urine was reduced to less than 2.0 by the use of 85% orthophosphoric acid. The use of orthophosphoric acid is justified as it is considered to be safe for human consumption and any carryover phosphoric acid in the distillate in reasonable amount is acceptable to the consumers, who hardly consume four tablespoons of RCUD per day.

The percentage of DNA strand break induced by MnO_2 and Cr^{+6} was 79% and 73% respectively on HPNLs against negative control (24%). Previous findings with RCUD at doses 1 µL/mL, 50 µL/mL and 100 µL/mL showed no genotoxic effect following 1 h exposure[4]. Furthermore, protective studies were carried out with the same doses of RCUD against MnO_2 and Cr^{+6}. Figures 1 and 2 show the protective effect of RCUD against manganese dioxide- and hexavalent chromium-induced DNA strand break in a dose dependent manner following pretreatment and simultaneous treatment. HPNLs on pretreatment with RCUD for 1 hour prior to MnO_2 and Cr^{+6} addition, showed a statistically significant level of protection in DNA strand break. However, when RCUD was added simultaneously along with MnO_2 and Cr^{+6}, the percentage of protection level was reduced compared to that of pretreatment.

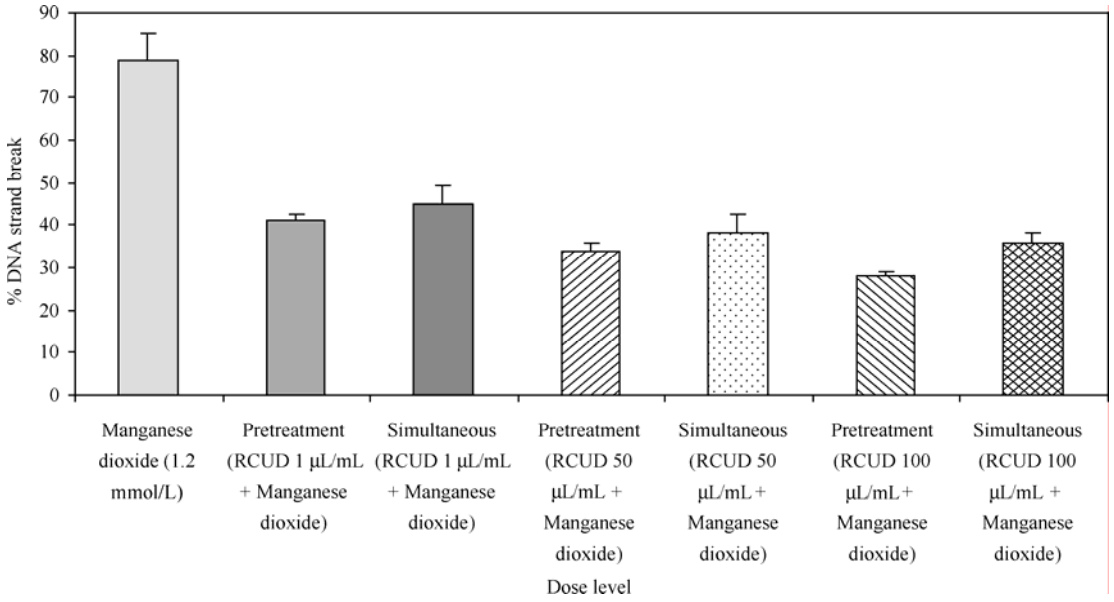

FIG. 1. Manganese dioxide (1.2 mmol/L) induced DNA strand break and the protective effects of RCUD pretreatment and simultaneous treatment on HPNL cells. The results are the mean of five sets of experiments ± standard deviation.

Table 1 and Fig. 3 show the anticlastogenic effect of RCUD on human lymphocytes (HLCs) against Cr^{+6} and MnO_2 exposure. When lymphocytes were treated with RCUD at 50 µL/mL and 100 µL/mL, no increase in the number of chromosomal aberrations and frequencies of micronuclei was observed as compared to the control (DMSO and sterile double distilled water), showing that RCUD had no clastogentic effect on HLCs. A statistically significant level of chromosomal aberration and frequency of micronuclei was observed on treatment with MnO_2 and Cr^{+6} alone at doses of 1.2 mmol/L and 5 µmol/L in human lymphocytes for 3 h. Human lymphocytes on treatment with RCUD 1 hour prior to the addition

of MnO_2 and Cr^{+6}, followed by 3-hour incubation, showed a significant reduction in mitotic index and frequency of micronuclei compared to simultaneous treatment (Tables 2 and 3, Figs. 4 and 5).

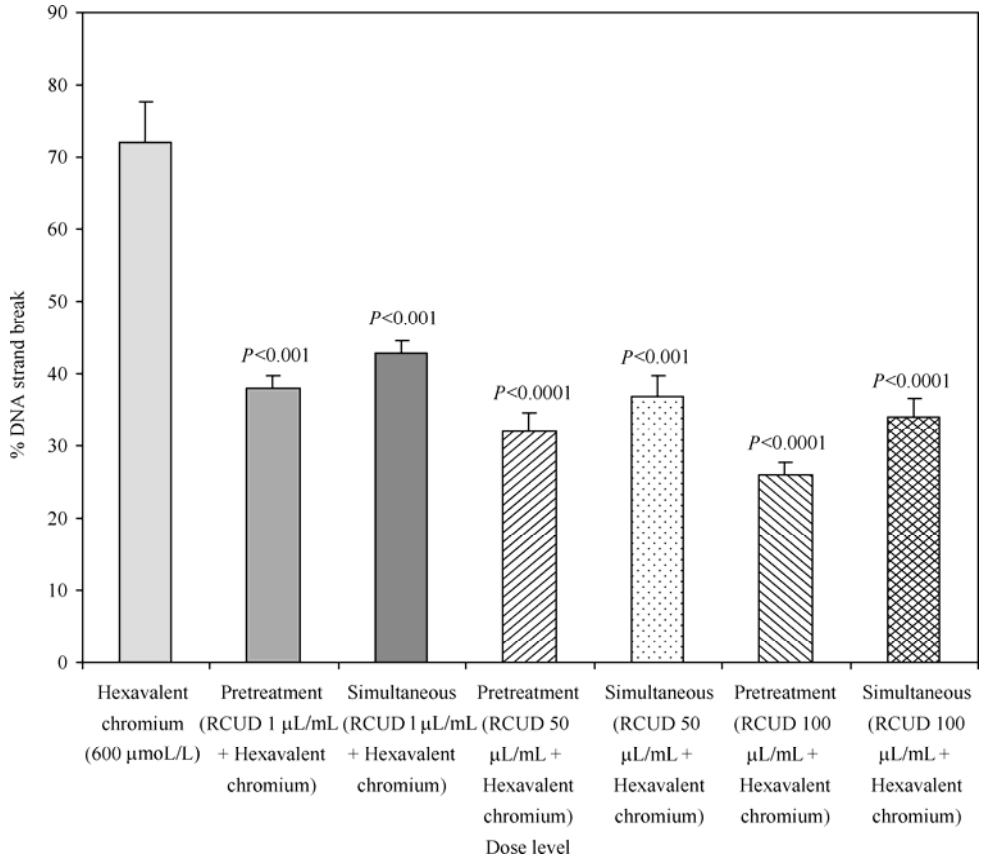

FIG. 2. Hexavalent chromium (600 µmol/L) induced DNA strand break and the protective effects of RCUD pretreatment and simultaneous treatment on HPNLs. The results are the mean of five sets of experiments ± standard deviation.

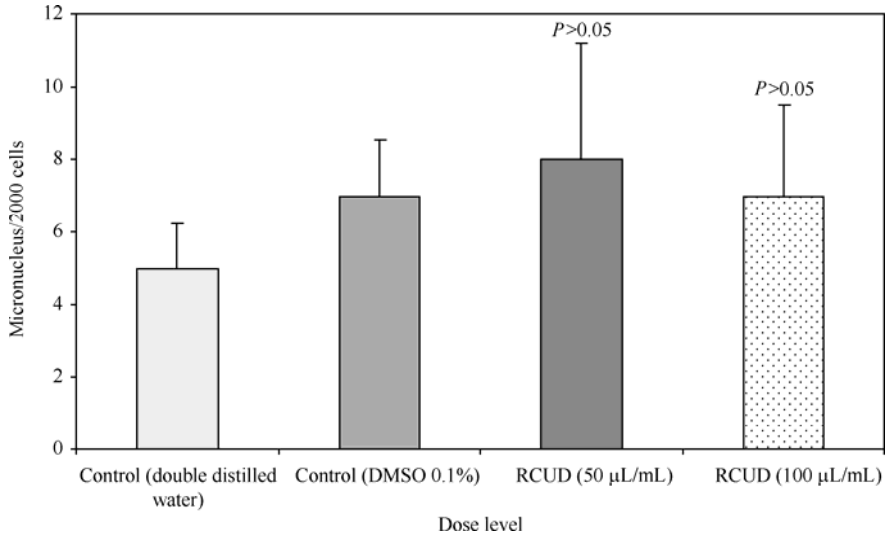

FIG. 3. Effect of RCUD on emergence of micronuclei in human lymphocytes. The results are the mean of five sets of experiments ± standard deviation.

TABLE 1

RCUD-induced Chromosomal Aberrations on Human Leukocytes

Dose	Metaphases (n)	% Aberrated cells ($\bar{x} \pm s$)	Aberration (n)	Types of Aberration						MI
				F	Gap	Ctb	Chb	Exc	Dic	
DMSO 0.1%	100	3.5 ± 0.707	4	2	ND	2	ND	ND	ND	5.9
Redistilled Cows Urine Distillate (RCUD) (50 µL/mL)	100	8.6 ± 4.04	10	2	4	3	1	ND	ND	5.4
Redistilled Cows Urine Distillate (RCUD) (100 µL/mL)	100	7.2 ± 1.08	8	3	2	3	ND	ND	ND	5.6

Note. The results are the mean of five sets of experiments ± standard deviation.

TABLE 2

Manganese Dioxide (1.2 mmol/L) Induced Chromosomal Aberrations and the Protective Effects of RCUD Pretreatment and Simultaneous Treatment on HLC

Dose	No. of Metaphases	% Aberrated Cells ($\bar{x} \pm s$)	Aberration (n)	Types of Aberration							MI
				F	Gap	Ctb	Chb	Exc	Dic	Pul	
MnO_2 (1.2 mmol/L)	100	46.4 ± 3.5	47	8	6	13	7	4	6	3	2.6
Pretreatment (50 µL/mL)	100	18 ± 2.124 ($P < 0.0001$)	18	4	3	4	2	ND	3	2	4.8
Pretreatment (100 µL/mL)	100	15 ± 2.369 ($P < 0.0001$)	15	3	4	4	2	ND	2	ND	4.8
Simultaneous Treatment (50 µL/mL)	100	23 ± 3.284 ($P < 0.0001$)	24	5	4	5	3	1	4	2	4.3
Simultaneous Treatment (100 µL/mL)	100	20 ± 4.325 ($P < 0.0001$)	18	3	4	4	3	ND	2	1	4.6

Note. The results are average of five sets of experiments. Decrease in values is statistically significant when compared to MnO_2 exposed cells. ND: not detected. Ctb: chromatid break. Chb: chromosome break. Exc: exchange. F: fragment. Dic: dicentric. Pul: pulverization. MI: mitotic index.

TABLE 3

Hexavalent Chromium (5 µmol/L) Induced Chromosomal Aberrations and the Protective Effects of RCUD Pretreatment and Simultaneous Treatment on HLC

Dose	No. of Metaphases	% Aberrated Cells ($\bar{x} \pm s$)	Aberration (n)	Types of Aberration						MI
				F	Gap	Ctb	Chb	Exc	Dic	
Cr^{+6} (5 µmol/L)	100	32 ± 3.3.005	40	6	6	13	8	4	3	2.6
Pretreatment (50 µL/mL)	100	14 ± 2.646 ($P < 0.0002$)	15	4	3	5	2	ND	1	4.8
Pretreatment (100 µL/mL)	100	10 ± 2.449 ($P < 0.0001$)	14	3	2	6	3	ND	ND	4.9
Simultaneous Treatment (50 µL/mL)	100	16 ± 1.708 ($P < 0.006$)	22	6	5	7	2	ND	2	4.6
Simultaneous Treatment (100 µL/mL)	100	13 ± 1.708 ($P < 0.001$)	17	5	4	5	2	1	ND	4.8

Note. The results are average of five sets of experiments. Decrease in values is statistically significant when compared to Cr^{+6} exposed cells. ND: not detected. Ctb: chromatid break. Chb: chromosome break. Exc: exchange. F: fragment. Dic: dicentric. MI: mitotic index.

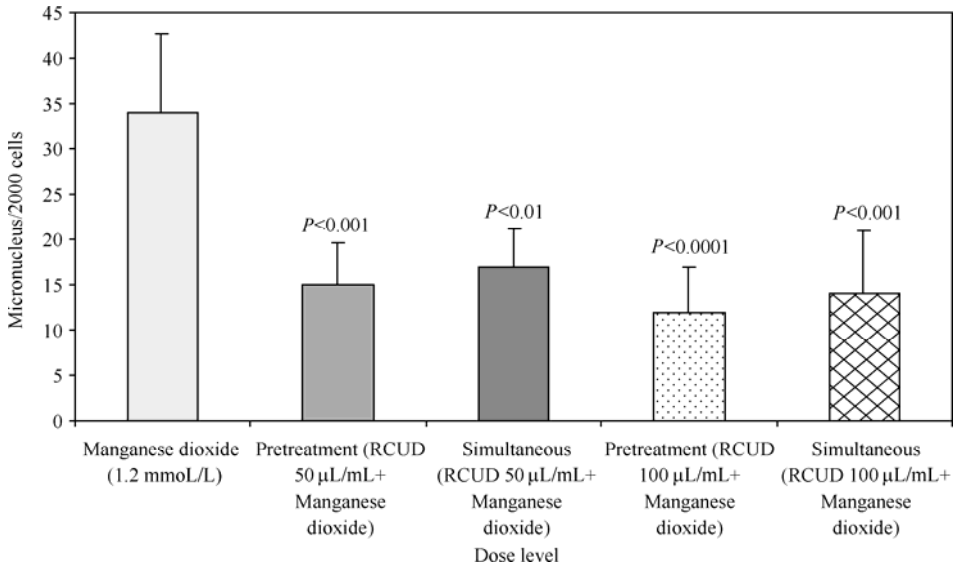

FIG. 4. Manganese dioxide (1.2 mmol/L) induced micronuclei and the protective effects of RCUD pretreatment and simultaneous treatment on HLC. The results are the mean of five sets of experiments ± standard deviation.

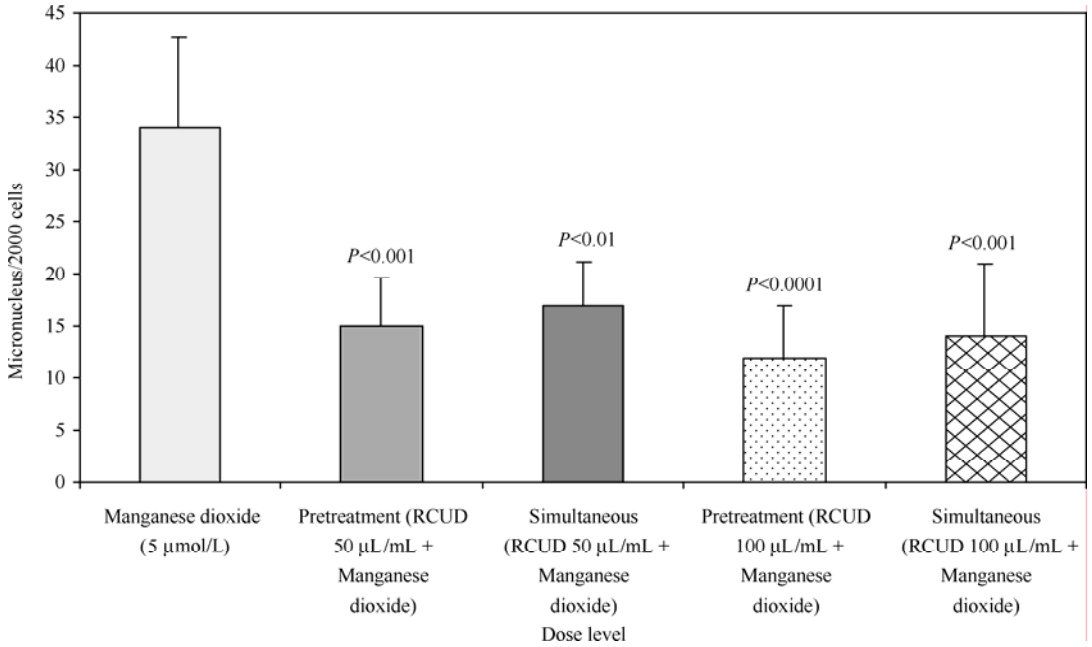

FIG. 5. Hexavalent chromium (5 µmol/L) induced micronuclei and the protective effects of RCUD pretreatment and simultaneous treatment on HLC. The results are the mean of five sets of experiments ± standard deviation.

DISCUSSION

It is well documented in the literature that Cr^{+6} causes DNA strand breaks and chromosomal aberrations *in vitro* and *in vivo*[12]. Cr^{+6} undergoes cellular reduction to a stable Cr (III) species [13-15]. In thecourse of reduction, reactive intermediates (ROS, sulfur-centered radicals, pentavalent and tetravalent chromium species) are generated, and 8-oxo-guanine (an indicator of oxidative DNA damage) is produced and excreted in urine[16-17]. Cr-induced genomic DNA damage includes formation of 8-hydroxydeoxyguanine (8-OH-dG, 7,8-dihydro-8-oxodeoxyguanosme), a form of oxidative DNA damage, Cr-DNA adducts, DNA-DNA interstrand crosslinks, single strand breaks and DNA-protein crosslinks. Hexavalent chromium reacts with hydrogen peroxide in cells to produce tetraperoxo-Cr^{+5}, which generates hydroxyl radicals by a Fenton-type reaction[18].

In the presence of hydrogen peroxide, Cr^{+6}

induces DNA single strand breakage, forms 8-OH-dG and produces a large amount of Cr-DNA adducts in the presence of gluathione. Glutathione thiol and hydroxyl radicals are reaction intermediates in Cr^{+6}-induced DNA damage[19].

The present investigation demonstrated that the known genotoxic/clastogenic effect of Cr^{+6} could be significantly modulated by the RCUD in both pretreatment and simultaneous treatment. The percentage of protection in DNA strand breaks was 47%, 56%, and 64% on pretreatment, and 40%, 49%, and 53% on simultaneous treatment at doses 1 µL/mL, 50 µL/mL, and 100 µL/mL respectively. Also, chromosomal aberration and micronuclei assays revealed that the modulatory effect was higher in case of pretreatment. The percentage of protection in the number of chromosomal aberrations on pretreatment was 56% with 50 µL/mL and 69% with 100 µL/mL, and 50% with 50 µL/mL and 59% with 100 µL/mL on simultaneous treatment. The frequency of micronuclei was 58% with 50 µL/mL and 67% with 100 µL/mL on pretreatment and the protection rate was 43% with 50 µL/mL and 55% with 100 µL/mL on simultaneous treatment. It has been reported that manganese compounds generate oxygen containing free radical intermediates and facilitate Fenton type reactions[7].

On pretreatment with RCUD against MnO_2-induced DNA strand break, the protection rate was higher in all assays. The protection rate was 48%, 67% and 65%, and 43%, 52% and 54% at 1 µL/mL, 50 µL/mL and 100 µL/mL on simultaneous treatment. For CA, protection rate on pretreatment with RCUD was 61% and 67% with 50 µL/mL and 100 µL/mL, and 56% and 65% with 50 µL/mL and 100 µL/mL for MN. Similarly, the RCUD exhibited statistically significant anticlastogenic effects at 50 µL/mL and 100 µL/mL on simultaneous treatment against MnO_2-induced CA and MN, showing 50% at 50 µL/mL and 56% at 100 µL/mL for CA and 50% with 50 µL/mL and 59% at 100 µL/mL for MN.

While mammalian cells *in vitro* express antioxidant enzymes such as SOD and catalase. These enzymes are expressed at much higher levels *in vivo*[20]. For example, catalase levels in cells from normal mouse liver were 1260±26 mU/mg protein and 58±3 mU/mg protein in cultured cells derived from the same tissue. Also the catabolic rates of compounds in RCUD in *in vivo* system were different from those in *in vitro* system, suggesting that *in vitro* mammalian cells are more vulnerable to oxidant-induced damage than *in vivo* mammalian cells. In order to resolve the possible inconsistencies between *in vitro* and *in vivo* studies, it is necessary to confirm the ameliorative effect of RCUD by conducting *in vivo* experiments.

It is hypothesized that, during pretreatment, the presence of RCUD prior to the addition of MnO_2 and Cr^{+6} simply prevents or reduces the formation of ROS. This may be due to the antioxidant property of RCUD attributed by the volatile acids and their derivatives identified using GC-MS[5], which scavenge the free radicals generated during the subsequent exposure of the cells to Cr^{+6} and MnO_2. However, in case of simultaneous treatment, the RCUD may react with the toxicant directly, thereby causing less protection against damage induced by the toxicants.

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