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ORIGINAL ARTICLE

The Positive Intervention Effects of Resveratrol on Acrylamide -Induced Cyto-/Genotoxicity in Primary Lymphocytes of Rat

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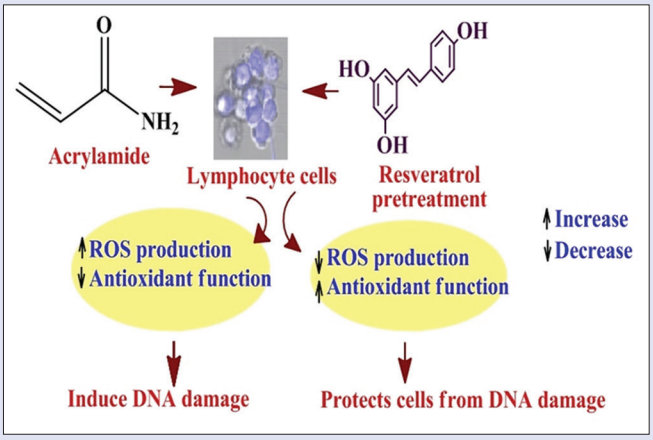
ABSTRACT

Background: Acrylamide (AA), a product formed from fried foods, has been recognized as a genotoxic agent in animals and humans. Oxidative stress is considered as an indirect cause of AA-related genotoxicity. **Objective:** Resveratrol (RES) (trans-3,4′,5-trihydroxystilbene) is a natural polyphenol produced by plants such as grapes, berries, and peanuts. In the present work, we studied the potential positive effect of RES in preventing oxidative stress-related cytotoxicity and genotoxicity induced by AA *in vitro* using rat primary lymphocyte cells. **Materials and Methods:** The primary lymphocyte cells were induced with AA (5–500 mg/L) for toxicological evaluation and pretreated with RES (100 μM) to determine its protective effect on AA-induced toxicity. The reactive oxygen species (ROS), an inference of oxidative stress, the antioxidant enzyme activity of catalase (CAT), superoxide dismutase (SOD) and glutathione S-transferase (GST) were measured. In addition, the micronucleus (MN) and nuclear fragmentation were analyzed by confocal microscopy to study the protective role of RES on AA-induced genotoxicity. **Results:** The cells pretreated with RES at a concentration of 100 μM for 24 h protected against AA-induced cytotoxicity, caused a reduction in ROS, showed an elevation in the activity of CAT, SOD, and GST signifying an increase in antioxidant function. Furthermore, the genotoxic effects of DNA damage, MN and nuclear fragmentation induced by AA were reduced on RES preexposure, thus showing the protective effect. **Conclusion:** Thus, our results suggested that RES effectively protected primary lymphocytes from AA-induced genotoxicity probably through reduction of ROS and increased antioxidant function.

Key words: Acrylamide, genotoxicity, lymphocyte cells, micronucleus, oxidative stress, resveratrol

SUMMARY

- Resveratrol found in grapes has shown to be protective toward genotoxicity induced by acrylamide, a compound formed in baked and fried foods studied *in vitro* in primary lymphocyte cells of the rat
- Acrylamide exerts genotoxic effects by increasing the reactive oxygen species levels, thus altering the antioxidant enzyme system (catalase, superoxide dismutase, and glutathione S-transferase) and exhibits DNA damage and micronucleus induction
- Resveratrol protected cells from genotoxic effects of AA by scavenging the free radicals resulting in the reduction of reactive oxygen species and increase in antioxidant function.



Abbreviations Used: AA: Acrylamide; RES: Resveratrol; ROS: Reactive oxygen species; SOD: Superoxide dismutase; GST: Glutathione S-transferase; MN: Micronucleus; CAT: Catalase.

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INTRODUCTION

Fried food that includes potato chips, French fries, bread, cereals, and coffee cooked at high temperature is considered as the prime source of human exposure to acrylamide (AA).[1] AA has also been used in industries in the manufacture of dyes, production of polyacrylamide which is used in papermaking, tertiary oil recovery and the manufacture of permanent press fabrics.[2] AA is known to exhibit genotoxicity, carcinogenicity and reproductive toxicity in rodents and is neurotoxic to humans and is categorized as the probable human carcinogen by the International Agency for Research on Cancer. The toxic effects of AA have been studied since the 1950s, but it gained the attention of scientist's after 2002.[3,4] The genotoxicity and cytotoxicity of AA sequel in the damage of the cellular oxidative defence system, inducing the release of reactive oxygen species (ROS) which is indicative of oxidative stress.[5] The enhanced oxidative stress caused by AA can result in DNA damage which might be associated with the depletion of an oxidative defence system.[6] In addition, the *in vitro* study of AA is known to induce

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sister chromatid exchanges, chromosomal aberrations, micronuclei, aneuploidy, and polyploidy, which is known to occur in the absence of metabolic activation to glycidamide.[7] Therefore, the genotoxicity of AA is considered necessary to be understood to control or reduce its toxicity.

Currently, many investigations are being carried out on antioxidants and other phytochemicals that play a significant role in either the prevention and cure of several illnesses, reduction of toxicity induced by chemicals or environmental factors.[8,9] The plants with high content of polyphenol and antioxidant activity such as *Theobroma cacao*, *Solanum nigrum*, *Panax ginseng*, *Digera muricata*, *Crocus sativus*, *Glycine max*, and *Zingiber officinale* and antioxidant compounds such as myrcitrin, genistein, and curcumin have been reported to be effective in the reduction of AA toxicity in cell lines and animal studies.[10,11]

Accordingly, these studies have shown that the inhibition of ROS formation, decrease in oxidative stress, increase in reduced glutathione (GSH) and reduction in cell apoptosis are general proposed mechanisms for protective effects of phytoconstituents against AA-induced toxicity.[12]

Resveratrol (RES), a polyphenol commonly found in grapes, has been shown to exert anti-inflammatory, antioxidant, antitumor in mammalian models studied.[13-15] It has also been implicated as cancer chemopreventive agent, competent to inhibit initiation, promotion, and progression of carcinogenesis induced by chemicals.[16] RES which plays a vital role as an antioxidant was studied *in vivo* and *in vitro* in cell culture models.[17,18]

Hence, an attempt has been carried in the present work to understand the protective role of RES on genotoxic effects caused by AA studied *in vitro* in peripheral blood lymphocytes of the rat. Oxidative stress factors, such as ROS and antioxidant enzymes were measured. Furthermore, the protection offered by RES on DNA damage induced by AA was analyzed.

MATERIALS AND METHODS

Chemicals and reagents

RPMI 1640 media, HiSep™ LSM, fetal bovine serum (FBS), phosphate buffer saline (PBS), trypsin, dimethyl sulphoxide, and quercetin were purchased from Himedia, India. AA, methyl thiazol tetrazolium (MTT) bromide, 2,7-dichlorofluorescein diacetate (DCFH-DA), penicillin/streptomycin, 4′,6-diamidino-2-phenylindole (DAPI), cytochalasin B, RES, 1-Chloro-2,4-dinitrobenzene (CDNB), bovine serum albumin (BSA) was purchased from Sigma-Aldrich Chemicals, India. Potassium chloride, trisodium citrate, citric acid, dextrose, TEMED (N, N, N, N-tetramethyl ethylenediamine), (ethylenediaminetetraacetic acid) were from SISCO Research Laboratories, India. All the other reagents and chemicals used in the present study were of analytical grade and were purchased from standard chemical companies.

Methods

Animal ethical statement

The animals (Swiss Albino Rats, CFTRI strain) were approved by the Animal Ethical Committee (IAEC N0. 348/14). The blood sample was collected as per the guidelines of the Ethical Committee.

Isolation of lymphocytes

Blood was collected aseptically from the Swiss Albino rats through heart puncture to the tubes containing a sterile anticoagulant, acid citrate dextrose solution A (22 g/L trisodium citrate, 8 g/L citric acid, and 24.5 g/L dextrose). The blood was diluted in the ratio of 1:2 with sterile PBS. Lymphocytes were isolated from blood using HiSep™ LSM LS001 as per the instructions in the manual. The buffy coat of lymphocytes isolated was washed with PBS. The cells were seeded in RPMI 1640 medium with 10% FBS. The viability of lymphocytes was checked using trypan blue exclusion test.[19]

Primary cell culture

The isolated lymphocytes were cultured as a suspension culture in RPMI 1640 medium with 10% FBS, 1% antibiotics (Penicillin and streptomycin) and were incubated in 5% CO_2 incubator with 95% humidity at 37°C and used for further experiments. All experiments mentioned below were carried out in triplicates.

Determination of viability of lymphocyte cell suspension by methyl thiazol tetrazolium assay

The lymphocyte cell suspension (1×10^5) grown in 96 well plate were exposed with different concentration of RES (25–200 μM) and AA (5–500 mg/L), respectively for 24 h. The positive effect of RES on AA-induced toxicity was studied by pretreating lymphocyte cell suspension for 24 h with RES at 100 μM concentration followed by AA (5–500 mg/L) treatment for 24 h. The untreated cell suspension was maintained as a control. The cell viability of all the treated and control cells was determined by MTT assay.[20]

Estimation of reactive oxygen species

Incubation of nearly 1×10^6 lymphocyte cell suspension cultured in 24 well plate with AA (5–300 mg/L) for 24 h and RES (100 μM) pretreated cells followed by AA-treatment for 24 h were analyzed for ROS by 2, 7-DCFH-DA.[21] DCFH-DA was added to the above-treated cell suspension at a final concentration of 5 μM and incubated for 40 min at 37°C in the dark. The fluorescence intensity of each treated and control cells was measured at an excitation and emission wavelengths of 485 and 550 nm, respectively (Varioscan Flash multimode plate reader, Thermo Scientific, Singapore).

Antioxidant enzyme activities

Treatment of lymphocyte cell suspension

An approximate of 3×10^5 lymphocyte cell suspension grown in 6 well plate containing 3 mL of RPMI 1640 medium were induced with AA (100, 200, and 300 mg/L) for 24 h at 37°C and was incubated with RES (100 μM) for 24 h before AA treatment. After incubation, all the above-treated cells were washed with PBS. Untreated cells were used as a control.

The above treated and control cells were lysed using lysis buffer (150 mM NaCl, 1% Triton X-100, 50 mM Tris pH 8.0) and were homogenized by sonication (sonicated for 20 s ON/5 s OFF at an amplitude of 50% for 6 rounds), supernatant was separated by centrifugation at 10,000 rpm for 10 min at 4°C. Total protein was quantified by Bradford assay with BSA as the standard.[22] The supernatant was used to estimate the enzyme activity of Catalase (CAT), superoxide dismutase (SOD) and GSH S-transferase (GST) and the absorbance was monitored in Varioscan Flash multimode plate reader, Thermo Scientific, Singapore.

Catalase activity

CAT activity was determined according to Aebi.[23] The enzymatic degradation of H_2O_2 was measured at 240 nm at 37°C, pH 7, and expressed as units/min/mg protein where one unit is the amount of enzyme required to decompose 1 μmole of H_2O_2/min.

Superoxide dismutase activity

SOD activity was estimated as described by Kostyuk and Potapovich[24] based on the inhibition of auto-oxidation of quercetin. The absorbance

was measured at 406 nm and expressed as the quantity of protein essential to inhibit 50% auto-oxidation of quercetin (1 unit) per mg protein.

Glutathione S-transferase activity

The activity of GST was determined by measuring the absorbance of conjugation of CDNB with reduced GSH at 340 nm as described by Habig *et al.*[25] The GST activity was expressed as nmol/min/mg protein with ε of 5.3 mM^{-1}.

Analysis of DNA damage by agarose gel electrophoresis

The DNA of AA treatment (100 and 200 mg/L) and RES (100 μM) pretreated lymphocyte cells were isolated by salting out, the method as described by Miller *et al.*[26] The cells were lysed and digested with SDS and proteinase K. This was followed by the addition of 6M NaCl and was centrifuged. The DNA was extracted from the supernatant by ethanol precipitation and checked for purity at an absorbance of 260 nm and 280 nm. The DNA isolated were analyzed for DNA damage by 1% agarose gel electrophoresis. The ethidium bromide stained DNA was photographed using Gel Doc system (Cleaver Scientific Ltd).

Micronucleus and nuclear fragmentation assay

For MN assay, cytochalasin B (3 μg/mL) was included in the culture medium in all the above-treated cells and was incubated for 6 h.[27] The cells were then washed with PBS, fixed in 10% formalin and stained with 100 nM of DAPI. The DAPI stained cells were observed under a confocal microscope (LSM 700, Carl-Zeiss, Germany, Zen software) for the micronucleus (MN) formation and nuclear fragmentation at the 60× objective lens and the frequency of MN were scored in 1000 binucleated cells.

Statistical analysis

Statistical analysis was done employing GraphPad Prism software. The experiments were carried out in triplicates**.** The data were expressed as means ± standard error of the mean and were analyzed by analysis of variance. $P < 0.05$ was considered as statistically significant.

RESULTS

Protective influence of resveratrol on cytotoxicity and reactive oxygen species formation induced by acrylamide in primary lymphocyte cells

The effect of RES on lymphocyte cells were studied by treatment of cells with RES at a concentration of 25–200 μM for 24 h. The lymphocyte cells treated with RES did not present cytotoxicity up to 100 μM and showed viability near to that of untreated control cells. However, at 200 μM concentration, a slight reduction in the viability of cells was observed. Hence, 100 μM concentration of RES was chosen for further studies [Figure 1a]. The study of cytotoxicity in AA-induced primary lymphocyte cells was observed to be concentration dependent. At 5 mg/L concentration of AA, the viability of cells was 94.55% and decreased gradually with increasing concentration of AA. At 500 mg/L of AA, cell viability was 46.84%. The best protective effect of RES was observed at 100 μM concentration of RES pretreatment on AA-induced cytotoxicity at concentrations of 100, 200 and 300 mg/L. Thus, further experiments of RES (100 μM) protection on AA-induction were carried at 100, 200, and 300 mg/L of AA-treatment [Figure 1b].

The ROS measured by DCFH-DA method in lymphocyte cells exposed to AA at concentrations of 5, 50, 100, 200 and 300 mg/L showed a concentration-dependent increase in DCF fluorescence intensity which is due to an increase in ROS formation. The RES (100 μM) preexposed cells followed by exposure to AA showed protection on

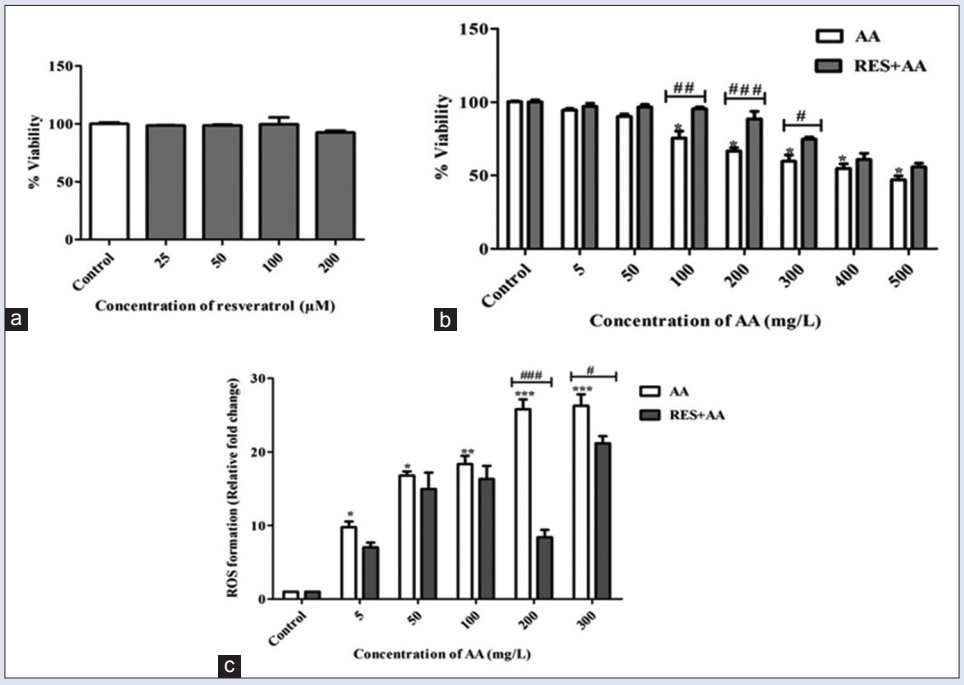

Figure 1: Effects of resveratrol on cell viability and reactive oxygen species formation in primary lymphocyte cells induced by acrylamide.(a) Effect of resveratrol (25–200 μM) on the viability of primary lymphocyte cells; (b) Effect of resveratrol preexposure (100 μM) on acrylamide-induced cytotoxicity; (c) Effect of resveratrol on reactive oxygen species formation in cells treated with acrylamide. All the values are ± standard error of the mean from experiment in triplicates. *$P < 0.05$, **$P < 0.01$ indicates significant difference between acrylamide versus control cells. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ indicates significant difference between resveratrol pretreatment versus acrylamide treated cells

AA-induced ROS formation, by reducing ROS formation with a decrease in DCF fluorescence intensity. The reduction of ROS was concentration-dependent. However, the RES preexposure at higher concentration of AA (300 mg/L) did not impart a good protective effect on ROS reduction [Figure 1c].

Resveratrol protected antioxidant enzyme activities (Catalase, superoxide dismutase, and glutathione S-transferase) in acrylamide exposed primary lymphocytes

The AA-induced lymphocyte cells showed a significant concentration-dependent decrease in the activity of CAT and SOD. The antioxidant enzymes CAT was reduced by 0.51, 1.75, 2.5-fold and SOD was decreased by 0.83, 1.27, 1.55-fold respectively at 100, 200, and 300 mg/L of AA-treatment in comparison to control cells. However, RES preexposed (100 μM), AA (100–300 mg/L)-treated lymphocytes showed an increase in CAT of 2.14, 2.1, 0.57-fold compared to AA-exposed lymphocyte cells. Hence, RES preexposure increases the decomposition of H_2O_2. In addition, the SOD activity was increased by 0.33, 0.5, 0.14-fold respectively to that of AA-exposed cells [Figure 2a and b].

The other antioxidant enzyme GST showed an increase of 0.84, 0.62, and 0.61-fold in AA-induced (100, 200, and 300 mg/L) lymphocyte cells compared to control cells. Furthermore, the RES (100 μM) pretreated followed by AA (100–300 mg/L) exposure showed an increase in GST activity to that of control by 1.27, 1.28, and 0.87–fold, respectively [Figure 2c].

The above result suggested that the RES was best protective at 100 and 200 mg/L of AA exposure. At 300 mg/L of AA, the protection offered by RES was decreased.

Resveratrol protects DNA of lymphocyte cells from acrylamide-induced DNA damage and nuclear fragmentation

Agarose gel electrophoresis of DNA isolated from lymphocyte cells pretreated with 100 μM of RES before AA treatment showed clear DNA band [lane 2, 3 of Figure 3a] similar to control cells [lane 1of Figure 3a]. However, the AA-induced cells at a concentration of 100 and 200 mg/L showed a characteristic DNA fragmentation observed as a ladder-like pattern as seen in lane 4, 5 of Figure 3a. Thus, the DNA was protected from damage induced by AA which signifies that RES protected cells from AA-induced DNA damage.

The confocal microscopy of lymphocyte cells exposed to AA (100, 200 mg/L) showed the formation of bi, trinucleate cells and nuclear fragmentation indicating the nuclei was observed to fragment into smaller pieces during AA exposure [Figure 3b]. However, RES (100 μM) preexposed cells, followed by the exposure to AA (100, 200 mg/L) were protected from nuclear fragmentation and were similar to control untreated cells. Thus, RES showed protection on nuclear damage induced by AA.

The protection offered by resveratrol on micronucleus formation in acrylamide-induced lymphocyte cells

The DAPI stained primary lymphocyte cells exposed to AA (100–300 mg/L), analyzed by confocal microscopy showed the formation of MN [Figure 3d]. The AA exposed lymphocyte cells showed an increase in the frequency of MN of 2.82, 6.29, and 8.79-fold at 100, 200, and 300 mg/L of AA treatment compared to untreated control cells. However, the lymphocyte cells preexposed to RES (100 μM) followed by AA (100–300 mg/L) exposure showed a decrease in the frequency of MN

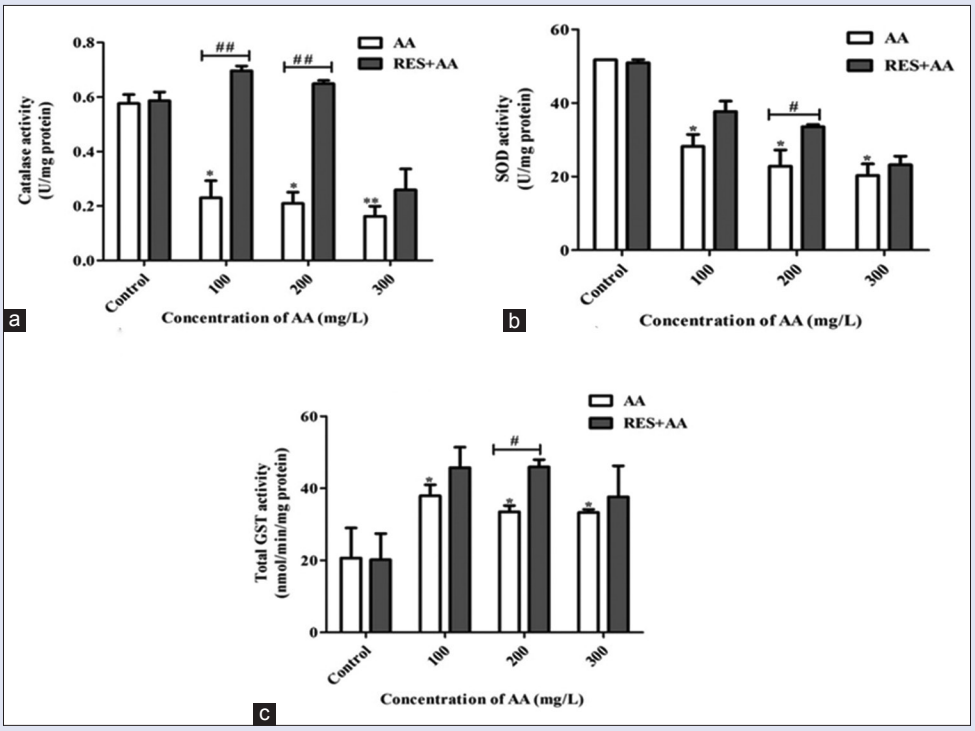

Figure 2: Effect of resveratrol on antioxidant enzyme activity in acrylamide exposed lymphocyte cells. (a) Catalase; (b) superoxide dismutase; (c) glutathione S-transferase. All value represents ± standard error of the mean from three independent experiments. The symbols indicates significant difference between acrylamide-induced and control cells (*$P < 0.05$), resveratrol pretreated and acrylamide-treated cells ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$)

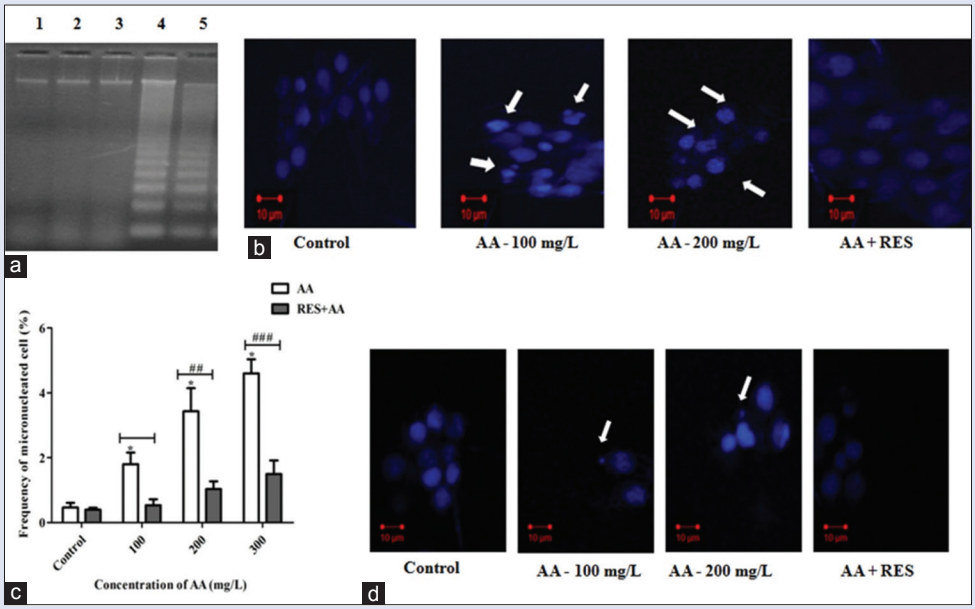

Figure 3: Protective effect of resveratrol (100 μM) pretreatment on (a) DNA fragmentation of lymphocyte cells induced by acrylamide resolved on 1% agarose gel electrophoresis; (b) Nuclear fragmentation induced by acrylamide analyzed by confocal microscopy; (c) % frequency of micronucleus; (d) confocal microscopy of acrylamide-treated and resveratrol pretreated cells for micronucleus formation. Experiments carried in triplicates are expressed as ± standard error of the mean. Significant difference of acrylamide-treated versus control cells (*$P < 0.05$), significant difference of resveratrol pretreated and acrylamide-treated cells ($^{\#}P < 0.01, 0.001$)

of 2.39, 2.33, 2.06 in comparison to AA-treated cells [Figure 3c]. Thus, signifies the protective effect of RES on AA-induced MN formation.

DISCUSSION

The natural genesis of AA formed during the cooking of food has resulted in identifying AA, as a cooking carcinogen.[28] Many studies have depicted the role of phytochemicals and antioxidants in the reduction of AA content in food sources.[12] Hence, there is a necessity to study the role of these antioxidants in reducing the toxicity induced by AA in animals and humans. RES found in plants has been shown to modulate oxidative changes in lung, liver, brain, kidney, and testes tissues of rat studied *in vivo*.[29] In this regard, lymphocytes from the blood of rat were used as an *in vitro* model to analyze the protective influence of RES on the genotoxicity of AA.

AA exposure to primary lymphocyte cells of rat exhibited apparent cytotoxicity at concentrations ranging from 5 to 500 mg/L. However, an increase in cell viability was observed with RES preexposure at 100 μM for 24 h which is a consequence of the reduction in cytotoxicity induced by AA. Hence, RES protected cells from AA-induced cytotoxicity. It has been reflected in our studies that AA exposed primary lymphocyte cells at concentrations of 100–300 mg/L have shown the increase in ROS level resulting in an increase in oxidative stress which might reduce the function of antioxidant enzymes. Hence, oxidative stress has been established to be responsible for inducing mutation, chromosomal aberration, promotion of a tumor, and development of cancer.[30]

The studies on RES were known to promote multiple antioxidant enzymes, such as SOD, CAT, heme oxygenase-1, a γ-glutamylcysteine synthetase, and NAD (P) H: quinone oxidoreductase.[31,32] The participation of antioxidant enzyme systems such as SOD and catalase has shown that SOD is involved in the dismutation reaction of the anion superoxide resulting in the formation of H_2O_2. H_2O_2 is then degraded by CAT forming H_2O and O_2 molecules, thus preventing the formation of hydroxyl radicals, thus prevents lipid peroxidation and known to shield against cellular damage as induced by free radicals.[33-35] As a consequence, the activities of these enzymes studied have shown that RES (100 μM) preexposed lymphocytes for 24 h showed a decrease in ROS resulting in a marked increase in the activities of CAT and SOD. Hence, RES can restore the antioxidant enzyme activity and probably can reduce the generation of free radicals and revealed a substantial protective effect against AA-induced oxidative stress to primary lymphocytes of the rat.

Furthermore, the previous studies have shown that RES marks an increase in the level of GSH, activities of GST, GSH peroxidase, and GSH reductase. The increased activity subsequently reduced DNA damage in various cells studied, such as Chinese hamster ovarian cells and human lymphocytes.[36,37] The GST, phase 2 detoxification enzyme activity studied in primary lymphocytes preexposed to RES followed by AA-exposure showed an increase in GST activity compared to AA exposed cells. Hence, GST could play a major role in the metabolic degradation of AA by conjugation with GSH.

The DNA has been shown to be sensitive to ROS induced by toxic chemicals. The increased ROS level marks the loss of balance of the scavenging system and thus can attack DNA macromolecules, resulting in oxidative DNA damage.[38] Hence, DNA damage was most likely through elevated intracellular ROS formation. The MN test an indication of chromosomal damage has shown an increase in the frequency of MN in AA (100–300 mg/L) exposed primary lymphocyte cells. The MN is formed from whole chromosomes when lags at anaphase or also exist from chromosome fragments at the phase of nuclear division.[39] Hence, AA acts as a genotoxic agent. However, preexposure of lymphocyte cells with RES was observed to be antigenotoxic by reducing the frequency of MN induced by AA. Furthermore, nuclear fragmentation of lymphocyte cells induced by AA was protected by RES pretreatment. The present work thus confirms the protective effect of RES on *in vitro* genotoxicity of AA.

CONCLUSION

The findings of our study imply that RES decreased ROS increased the activities of CAT, SOD, and GST enzymes. Thus, RES reduced oxidative stress-related genotoxicity induced by AA in lymphocyte cells. In addition, RES is protective on AA-induced DNA damage and can be contemplated in the prevention of genetic risk associated with the exposure of AA.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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