ISSN: 0975-833X

Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 8, Issue, 06, pp.33006-33018, June, 2016

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

RESEARCH ARTICLE

PROTECTION OFFERED BY RESVERATROL AGAINST ACRYLAMIDE INDUCED GENOTOXICITY IN *C. ELEGANS*

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ARTICLE INFO

Article History:
Received 28th March, 2016
Received in revised form
23rd April, 2016
Accepted 10th May, 2016
Published online 30th June, 2016

Key words:

Caenorhabditis elegans,
Acrylamide, genotoxicity,
Dose-response,
Resveratrol, protection.

ABSTRACT

Acrylamide has been shown to be neurotoxic, genotoxic and a carcinogenic compound formed during the frying or baking of foods by means of the Maillard reaction. To evaluate the biological consequences of continuous exposure to acrylamide at levels found in common foodstuffs and its protection by resveratrol, we studied the effects of acrylamide using an *in vivo* model, nematode *Caenorhabditis elegans*. As for growth and fecundity nematodes showed retarded growth with reduced body and brood sizes, at 500 mg/L of acrylamide. Lifespan decreased significantly even in 500mg/L of acrylamide. The genotoxic effect was observed to be heritable being carried to further generations. Acrylamide formed DNA adducts bringing about the genotoxic effect. Of the xenobiotic metabolic genes studied, *cyp*-35A2, *ugt*-44 were upregulated in acrylamide-exposed worms. Among DNA repair genes studied *xpa*-1, *apn*-1, *rpa*-1, *msh*-2, *ddb*-1 and *exo-3* were downregulated. Resveratrol which is a naturally occurring polyphenol exhibited protection against AA toxicity on growth, fecundity, and lifespan. The genotoxic effect of acrylamide was protected by resveratrol. The resveratrol preexposed worms showed upregulation of metabolic genes *gst*-4, *ugt*-44, a life span longevity genes *daf*-2 and DNA repair genes *xpa*-1, *apn*-1, *rpa*-1, *msh*-2, *ddb*-1.

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Citation: Ranjini, A., Chinnu Salim, Rajini, P. S. and Manonmani, H. K. 2016. "Protection offered by Resveratrol against Acrylamide induced Genotoxicity in *C. elegans*", *International Journal of Current Research*, 8, (06), 33006-33018.

INTRODUCTION

Acrylamide (AA) is an odourless white crystal, soluble in water, ethanol, ether, chloroform with a chemical formula C_3H_5NO. Its IUPAC name is prop-2-enamide. Polyacrylamide, the nontoxic AA polymer is commonly used in chemical, paper, fiber, plastic industries and is used as soil stabilizers, in gel electrophoresis, and so on. In contrast, the AA monomer, a known industrial hazard has shown to exhibit neurotoxicity in both animals and humans, carcinogenicity in experimental animals and mutagenicity in somatic and germ cells (Friedman, 2003). However, AA has been found in the recent investigation, in the fried and baked foods which is formed by Maillard reaction (Friedman, 2003; Tareke *et al*., 2002). It is present in common foods like bread, baked cookies, fried potato, roasted nuts, roasted coffee, etc. (Mohan Kumar *et al*., 2012). This finding of AA in food has significantly raised public concerns of AA's potential risk to the health of people upon dietary exposure to the chemical. AA monomer has been classified as a Group 2A carcinogen by the International Agency for Research on Cancer (IARC, 1994). Importance is sited on AA genotoxicity and tumorigenicity with a special focus on DNA adduct targeted mutagenesis, which is a vital area of research (Besaratinia *et al*., 2005). Many of the research studies confirmed that AA and its biotransformed metabolite, glycidamide (GA) are neurotoxins, reproductive toxins, and carcinogens in addition to their hazard for other organs like the lungs, liver, kidney and intestines (Capuano and Fogliano, 2011). AA and GA can form covalent adducts with haemoglobin, DNA, and other functional groups of proteins like SH, inducing chromosomal aberration, gene mutation, increasing benign and malignant neoplasm in rodents (Kalita *et al*., 2013; Pelucchi *et al*., 2011). The exposure of both AA and GA were observed to be mutagenic in the liver cII gene and lymphocyte Hprt gene of big blue mice (Manjanatha *et al*., 2006). *Caenorhabditis elegans* (*C. elegans*), a whole animal system has been used for toxicological research to analyze various biological parameters such as growth, body movement, lifespan and reproduction (Miwa *et al*., 1982; Tabuse *et al*., 1989). *C. elegans* exposed to AA of concentrations commonly found in daily consumed

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foodstuffs showed no deleterious effects on growth or fecundity (Hasegawa *et al*., 2004b). The DNA microarray data showed that genes for some major detoxification enzymes, such as GST (glutathione S-transferases), UGT (uridine diphosphate-glucuronosyl/glucosyl transferases), and SDR (short-chain type dehydrogenases) were upregulated more than twofold by the AA exposure (Hasegawa *et al*., 2008).

The importance of finding an effective way to minimise AA hazards, several research have been carried out on plant extracts and the phytochemicals on different aspects of AA toxicity. Plants with antioxidant compounds like genistein, myrcitrin, resveratrol, and curcumin have been reported to be effective in the reduction of AA toxicity in cell lines and animal studies (Zhang *et al*., 2007 a,b). Resveratrol, a trans-3,5,4'-trihydroxystilbene is a phytoalexin found in many plant species which includes those often consumed by humans such as grapes, berries, and peanuts. It is produced in plants in response to mechanical injury, fungal infection, and UV radiation (Langcake, 1976). Resveratrol showed protective effects against cardiovascular diseases, cancer, and neurodegenerative diseases, because of its antioxidative, antimutagenic, and anti-inflammatory activities (Zhuang *et al*., 2003; Baur and Sinclair, 2006). Resveratrol administration has been shown to increase the lifespan of yeast by 70% (Howitz *et al*., 2003). Also prolonged the lifespan in *C. elegans*, *Drosophila melanogaster* (Wood *et al*., 2004), lower vertebrates (Valenzano *et al*., 2006) and mice (Baur *et al*., 2006). The other mechanism by which resveratrol exerts its beneficial effects may be due to its ability to attenuate oxidative DNA damage (Guo *et al*., 2007). But, whether resveratrol acts by enhancing DNA repair or preventing oxidative DNA damage is currently unknown. Hence, resveratrol was chosen in our studies to understand the protective effect of resveratrol against AA-induced toxicity in *C. elegans* on various biological parameters such as growth, reproduction, body movement and lifespan. We also attempted to address the DNA adduct formation exposed to AA and heritability, the fate of expression of certain genes of AA exposure and protection caused by resveratrol.

MATERIALS AND METHODS

Acrylamide, resveratrol, nuclease P1 were purchased from Sigma Chemicals, India. Alkaline phosphatase, acid phosphatase, proteinase K, tris HCl from SRL chemicals, India. RNase A, CTAB, were purchased from Himedia, India. dNTP, Taq polymerase from Genie, Bangalore. RNA isolation kit was purchased from GE Healthcare, India. cDNA synthesis kit from Thermo Scientific, India and Syber green (Fast start Essential DNA Green Master) from Roche Diagnostics, Germany.

Primers for RT-qPCR: Primers were designed based on the sequences retrieved from the NCBI database. *ugt-44* (NM_069532.4), *gst-2* (NM_069446.3), *gst-4* (NM_0694 47.6), *sdz-8*(NM_073520.1), *msh-2(*NM_058801.5), *apn-1*(NM_063286.5), *exo-3* (NM_001026414.3), *xpa-1* (NM_05 9624.1), *rpa-1* (NM_063205.4), *ddb-1* (NM_069898.7). *Primers for cyp35A2* (Shu-hua *et al.,* 2011), *sod-3, daf-2, daf16* (Li *et al.,* 2008) were taken from respective literature reports. Primers were synthesized by Sigma-Aldrich Chemical Company, Bangalore, India. Primers used in the study are given in (Table 1). All other chemicals that were used were of analytical grade.

Methods

C. elegans culture and maintenance

The strain of *C. elegans,* the wild-type strain N_2, and *Escherichia coli* OP50 (*E. coli* OP50), an uracil auxotroph was obtained from the Caenorhabditis Genetics Center (CGC, Minneapolis, MN, USA), which is funded by the National Center for Research Resources (NCRR).

Worm culture and Maintenance

Worms were cultivated on nematode growth medium plates (NGM) with OP50 strain of *E. coli* and maintained at 20^{o}C (Brenner, 1974; Williams *et al*., 1990). Synchronization of worms was done by preparing the eggs from gravid adults by sodium hypochlorite treatment (Williams *et al*., 1990) .The synchronous eggs were allowed to develop on NGM plates. The worms were washed out of the plate by using K-medium (50 mM NaCl, 30 mM KCl).

Preparation of Acrylamide

5 mg of pure acrylamide was dissolved in 10 ml of sterile milliQ water and serially diluted using sterile milliQ water.

Resveratrol stock solution

1mM stock solution was prepared in DMSO (2% in sterile water) and was used at 100µM concentration.

Experimental conditions

Age synchronized worms (L4 stage) were exposed to sub lethal concentrations (500, 1000 , 2000 mg/L) of AA in 1 ml K-medium in a 12-well tissue culture plate along with *E. coli* OP50 and incubated for 24 h at 20ºC for dose determination with certain physiological parameters (Brenner, 1974). Later the nematode exposed to 500 mg/L of AA was taken for further studies of co-exposure with resveratrol. After the exposure period, the worms were taken, washed thrice with K-medium and used for various experiments.

Analysis of behavior parameters

Egg laying

After the exposure period, 10 worms from each group were taken in NGM plates with *E. coli.* The number of eggs laid by each worm during 1 h after exposure was counted (Bany *et al*., 2003). Experiment was repeated 3 times in triplicates.

Brood size

After the co-exposure, a single worm was picked and transferred to 12-well tissue culture plates containing 1 ml K-

medium, with *E. coli* OP50 at a dilution of 1 OD at 550 nm. The plates were kept at 20 °C for 72 h. After 72 h, the worms were washed, pelleted, and the progeny was counted (Middendorf, 1993). Assays were repeated 3 times in triplicates.

Locomotion

For analysis of locomotion, after the exposure period, worms were transferred to NGM plates. The worms were allowed to adapt for 10 min and then the locomotive rate was quantified (Ali *et al.,* 2012; Sawin *et al.,* 2000). Ten worms from each group were studied, and the assay was repeated three times in triplicate.

Lifespan

After the exposure period, worms were washed with K-medium and 20 ± 1 L4 worms were taken in a 24 well plate containing K-medium with *E. coli* OP50 as food and 5-fluoro-2-deoxyuridine (FudR 50 µM). The worms were maintained at 20°C and checked for survivability every day until all the worms died (Keaney *et al.,* 2004).

DNA adduct formation

L1/L2, L3/L_4 and Gravid stage of *C. elegans* grown on NGM agar medium containing *E.coli* OP50 were washed with K-media. 500 worms in K-media were treated with AA at a concentration of 0.0005 mg/L, 0.005 mg/L, 0.05 mg/L, 0.5mg/L, 5 mg/L, 50 mg/L, 500 mg/L. *E coli* OP50 was added and incubated for 24 h at 20°C. After incubation, the worms were washed with K-media to remove *E coli*. Total DNA was isolated from the worms of each treatment by transferring 500µl aliquot of worms to 4.5 µl worm lysis buffer (0.1M Tris-HCL, 0.1M NaCl, 50mM EDTA pH 8, 1% SDS) and 20 mg/ml of proteinase K, incubated at 62º C for 60 minutes. After incubation 800 µl of 5M NaCl was added, mixed by inversion, 800 µl of 10% CTAB in 0.7M NaCl was added, incubated at 37ºC for 10 minutes. 7ml of Tris (pH 8) saturated Phenol/chloroform/isoamyl alcohol (25:24:1) was added, mixed and spun to recover aqueous phase. Then 10 µl of 10 mg/ml of RNase A was added and incubated for 2 h at 42º C.

The aqueous phase recovered was added with 100 µl of 3M sodium acetate and an equal volume of 100% cold ethanol, inverted to mix. A stringy white DNA precipitate obtained was kept at -20º C for 24 h. After 24 h the above solution was centrifuged, the DNA pellet was washed with 70% ethanol, air dried and dissolved in water. DNA was quantified spectrophotometrically at 260/280 nm (Nanodrop, Eppendorf). The isolated DNA was subjected to thermal hydrolysis at 100°C followed by enzymatic hydrolysis at 37°C for 24 h, using nuclease P1 (24 U/ml; phosphodiesterase), alkaline phosphatase (2.4 U/ml), acid phosphatase (0.3 U/ml). The enzymatic hydrolysis was stopped by heating the sample at 100°C for 5 min, followed by centrifugation and filtration to remove the denatured enzyme protein (Beranek *et al*., 1980). The enzymatically hydrolyzed DNA sample was subjected to LC-ESI/MS/MS analysis (Gamboa da Costa *et al*., 2003) to identify the adduct formation.

RNA isolation and RT-qPCR

Gravid stage of *C. elegans* at a density of 500 worms grown on NGM agar medium containing *E. coli* OP50 were treated with AA at a concentration of 500 mg/L for 24 h. Worms at a density of 500 were pretreated with resveratrol at a concentration of 100µM and were incubated for 24 h. After incubation, worms were washed with K-media and treated with AA at a concentration of 500mg/L and incubated for another 24 h. The worms from each treatment were washed with K-media till *E. coli* OP50 was completely removed. Total RNA was isolated from the worms of each treatment by using GE Healthcare RNA isolation kit according to manufacturer's instructions. Extracted RNA was quantified spectrophotometrically at 260/280 nm (Nanodrop, Eppendorf) and the integrity was checked using a 1% agarose gel. cDNA was synthesised, using Thermo Scientific First strand cDNA synthesis kit according to manufacturer's instructions. cDNA synthesized was stored at 20°C for further use. RT-qPCR reactions were performed using C1000 Touch thermal Cycler (Biorad CFX96) using Syber Green (Fast start Essential DNA Green Master) with cDNA at a concentration of 20 ng and 400 nmol of forward and reverse gene-specific primer. The RT-qPCR conditions were 95°C for 5 min, 95°C for 20 sec, 62° for 40 sec annealing temperature, 72°C for 20 sec, 45 cycles. For RT-qPCR, *act*-1 was used as the internal control, and the RNA level of each gene of interest was normalized to the level of *act*-1 for comparison. The results of RT-qPCR experiment are from triplicate in three repetitions.

Protective effect of resveratrol on AA-induced gene sequence variation in *rpa-1 (replication protein a-1)* gene of *C. elegans*

F0, F1 and F2 generation of gravid stage worms at a density of 500 worms grown on NGM agar medium (supplemented with *E.* coli OP50) were used for treatment with AA. The worms were pretreated with resveratrol before AA treatment at a concentration of 100µM to a density of 500 worms and were incubated for 24 h. After incubation worms were washed with K-media and treated with AA at a concentration of 500mg/L and incubated for another 24 h. The worms from each treatment were washed with K-media till *E. coli* OP50 was completely removed. Total DNA was isolated from each treatment and from without treatment controls of F0, F1, and F2 generation of *C. elegans*. The DNA was quantified spectrophotometrically at a wavelength of 260/280 nm (Nanodrop, Eppendorf).

Polymerase chain reaction (PCR)

PCR was conducted with dNTP, Taq polymerase and Taq buffer, 100nm of forward and reverse primer of *rpa-1* gene. The primer sequences of the *rpa-1* gene: Forward primer: GAAGATCGCTACCCTTCTCA; Reverse primer: GTCATCGGATTCCTCCTTTG (Neher *et al.,* 2006) was used to amplify *rpa*-1 (synthesized by Sigma-Aldrich Chemical Company, Bangalore, India). Genomic PCR was performed over 30 cycles (denaturation: 94 °C for 45 s; annealing: 59 °C for 60 s; extention 72 °C for 60 s). The amplified *rpa*-1 gene

was analyzed by electrophoresis on 1.5-2% agarose gels containing ethidium bromide.

DNA sequencing

The amplified *rpa*-1 gene of control, AA treated, Resveratrol pretreated followed by AA treatment were sequenced at Xcelris Labs Ltd. The *rpa*-1 gene sequence of each treatment and control obtained was checked for homology using Multiple Sequence Alignment CLUSTAL W and mutations were analysed using the software Genious.

RESULTS

Effect of different concentrations of AA on egg laying

The presence of AA had deleterious effect on number of eggs laid per worm. There was concentration dependent decrease in the number of eggs laid per worm (Fig 1a). There were 24%, 35% and 54% decrease in the number of eggs laid per worm at concentrations of 500, 1000 and 2000mg/L of AA respectively.

Effect of different concentrations of AA on brood size

The presence of AA had detrimental effect on brood size. The decrease in the progeny was concentration dependent. There was 19, 50 and 56% decrease in the number of progeny per worm at 500, 1000 and 2000mg/L concentration of AA respectively (Fig 1b).

Effect of different concentrations of AA on locomotion

The AA exposed worms showed concentration dependent decrease in the motility. There was 63%, 56% and 55% decrease in bodybends/20 secs in the worms treated at concentrations of 500, 1000 and 2000mg/L of AA respectively (Fig 1c). Of these concentrations 500mg/L concentration of AA was chosen because detectable aberrations were observed at this level and the changes were not linear at higher concentrations.

DNA adducts formation in *C. elegans* exposed to different concentrations of AA

Gravid stage of *C. elegans* exposed to different concentrations of AA (0.0005 mg/L, 0.005 mg/L, 0.05 mg/L, 0.5mg/L, 5 mg/L, 50 mg/L, 500 mg/L) showed DNA adduct formation (Table 2). Adenine, thymine and guanine nucleotide bases showed adduct formation except cytosine, where the adduct of cytosine, N_3N_4 (2-hydroxypropanoyl) deoxycytidine was observed to be formed only at 500 mg/L of AA. Also, more adducts were found to be formed at 500 mg/L of AA concentration. Hence, the concentration of 500 mg/L of AA appeared to be the best for the formation of DNA adducts.

DNA adduct formation at different stages of growth of *C. elegans*

AA was exposed to different growth stages of *C. elegans*. It was observed that gravid stage was more susceptible showing more number of adducts (Table 3). The number of adducts was increased with increase in age of the worm. Hence gravid stage was chosen for further studies.

Effect of resveratrol on AA toxicity

In preliminary experiments, different concentrations (50, 100 and 200 µM) of resveratrol were used for studying their preventive effect on AA-induced toxicity. AA was used at 500mg/L concentration. Of these concentrations, 100 and 200 µM concentration of resveratrol showed similar results (data not shown). Hence, 100 µM concentration of resveratrol was used in all the studies.

Effect of resveratrol on AA toxicity on egg laying

The animals in the AA containing plates showed a reduction in the number of eggs laid as compared to the control. Resveratrol *per se* did not impart a noticeable effect on egg laying per worm. Resveratrol pretreatment followed by AA exposure protected the reduction in egg laying by 11 % (Fig 2a) i.e. there was 1.11 fold increase in egg laying in resveratrol pretreated worms compared to AA treated worms.

Effect of resveratrol on AA toxicity on brood size

The animals treated with AA showed a reduction in the number of progeny as compared to the control. Resveratrol did not impart a noticeable effect on brood size of the worm. Resveratrol pretreatment followed by AA exposure protected the decrease in the number of progeny by 1.41 folds i.e. there was 22% increase in brood size after resveratrol pretreatment followed by AA exposure (Fig 2b).

Effect of resveratrol on worm locomotion

We performed AA-induced toxicity trials by observing the effects of AA on motility, as the impairment of worm movement is a readily observable which is a consequence of AA exposure in worms. We chose concentration of AA (500mg/L) that produced observable toxicity as explained above. At the end of exposure, the motility of the AA exposed worms was reduced by 42% compared to unexposed control worms (Fig 2c). Worms preexposed to 100µM of resveratrol for 24h followed by AA exposure (for 24h) showed a protective ability of resveratrol towards locomotive toxicity. The movement was restored by 28% to that of AA exposed worms. Resveratrol did not exhibit noticeable neurological impairments on *C. elegans.*

Effect of Resveratrol on life span of *C. elegans*

The life span of *C. elegans* was reduced to 24 days by AA exposure which was a reduction of 4 days. Resveratrol pretreatment increased the life span by 3 more days in AA-treated worms (Fig 3) i.e. there was an enhancement of 17.24 % in life span due to resveratrol protection.

Expression studies on selected metabolic and DNA repair genes in the nematode *C. elegans*

Though the worms resumed normal behaviour based on visual observations in resveratrol preexposure group, we attempted to study the gene expression of toxicity relevant genes throughout normal development in both AA toxicity induced, and resveratrol protected worms.

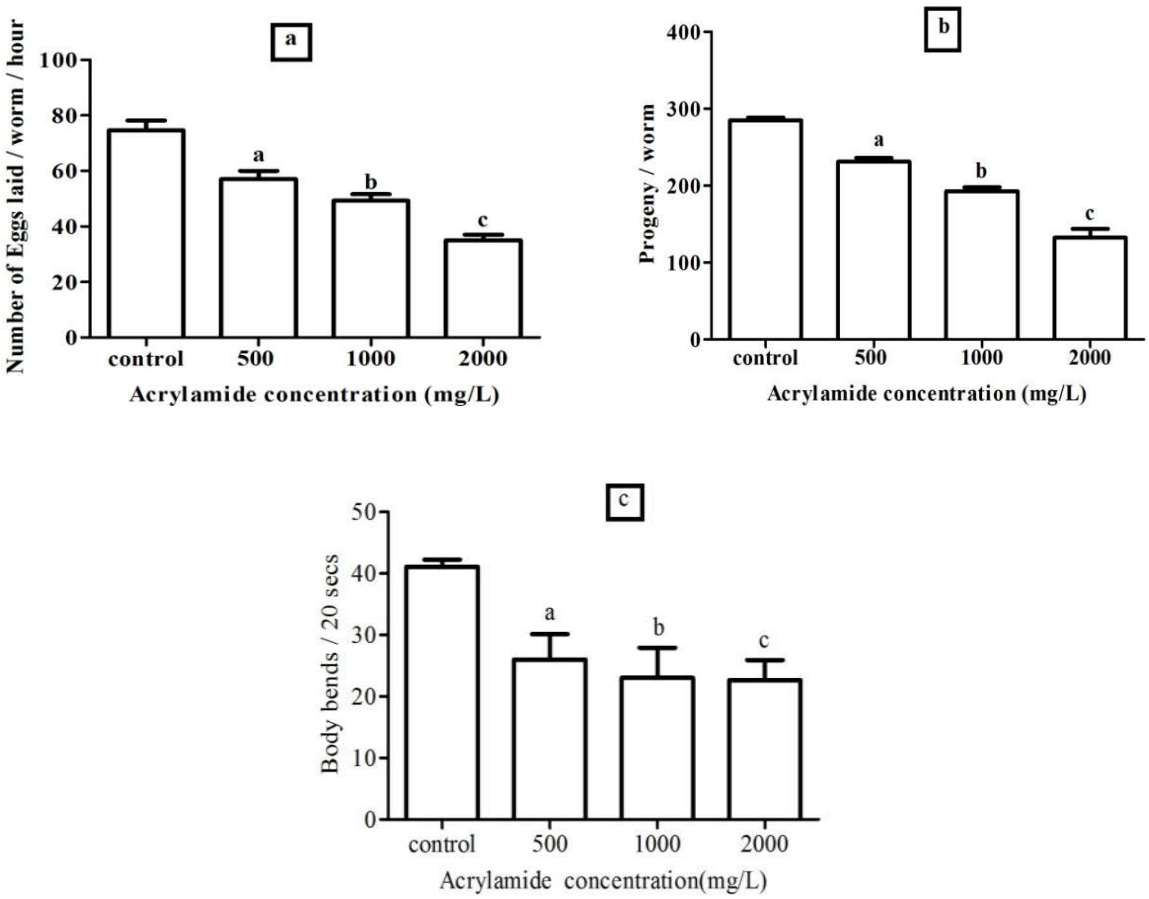

Fig 1. Effect of AA on behaviour parameters of worms exposed to AA

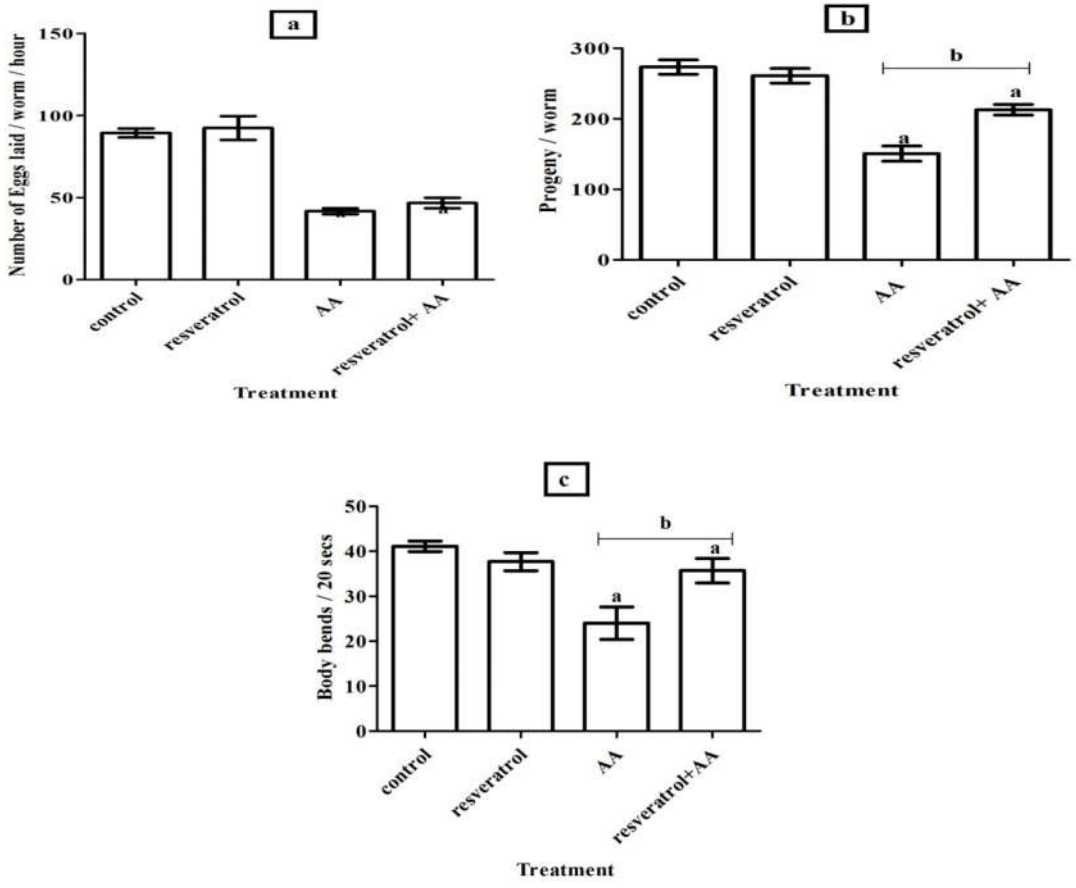

Fig 2. Effect of Resveratrol on AA treated worms on behaviour parameters

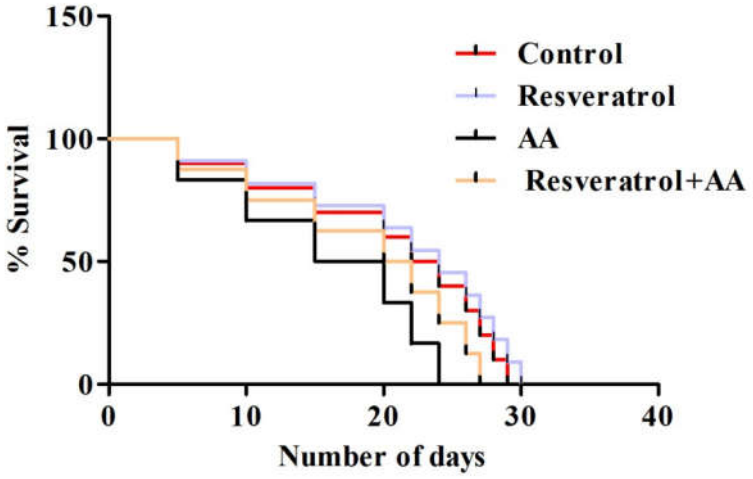

Fig 3. Survival curves of *C. elegans* in different treatment groups

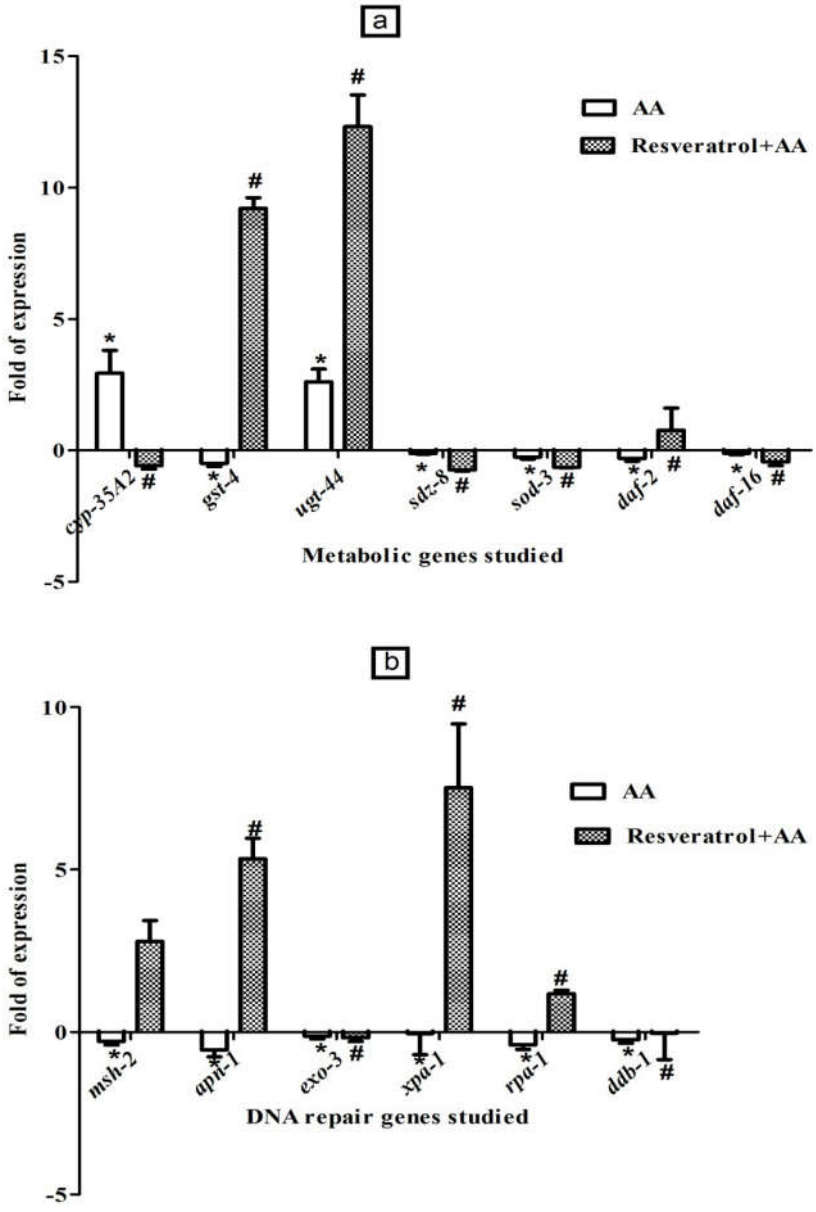

Fig 4. Effect of Resveratrol on AA treated worms on expression of few metabolic and DNA repair genes

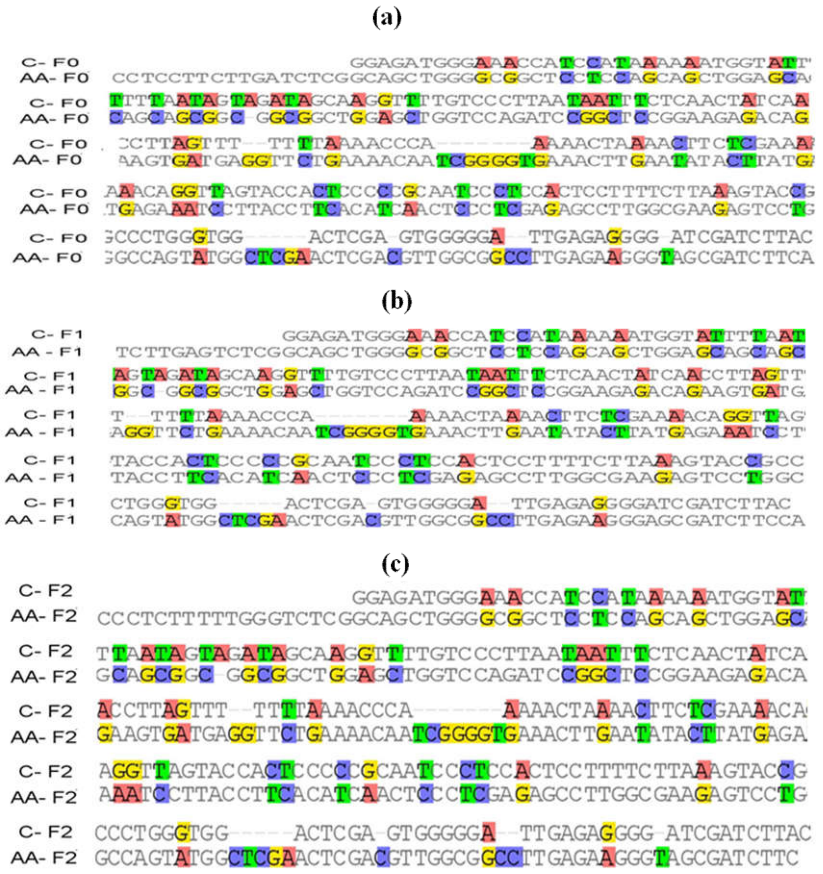

Fig 5. Transition mutations in *rpa-1* gene of nematode *C. elegans* generation exposed to AA

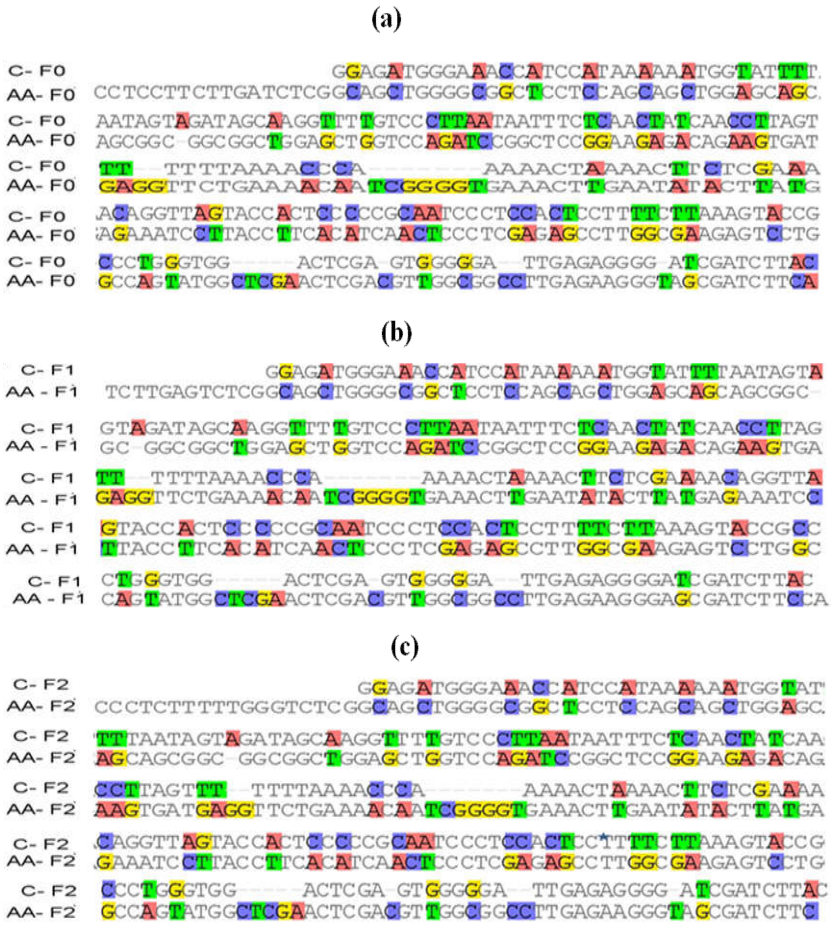

Fig 6. Transversion mutations in *rpa*-1 gene of nematode *C. elegans* generation exposed to AA

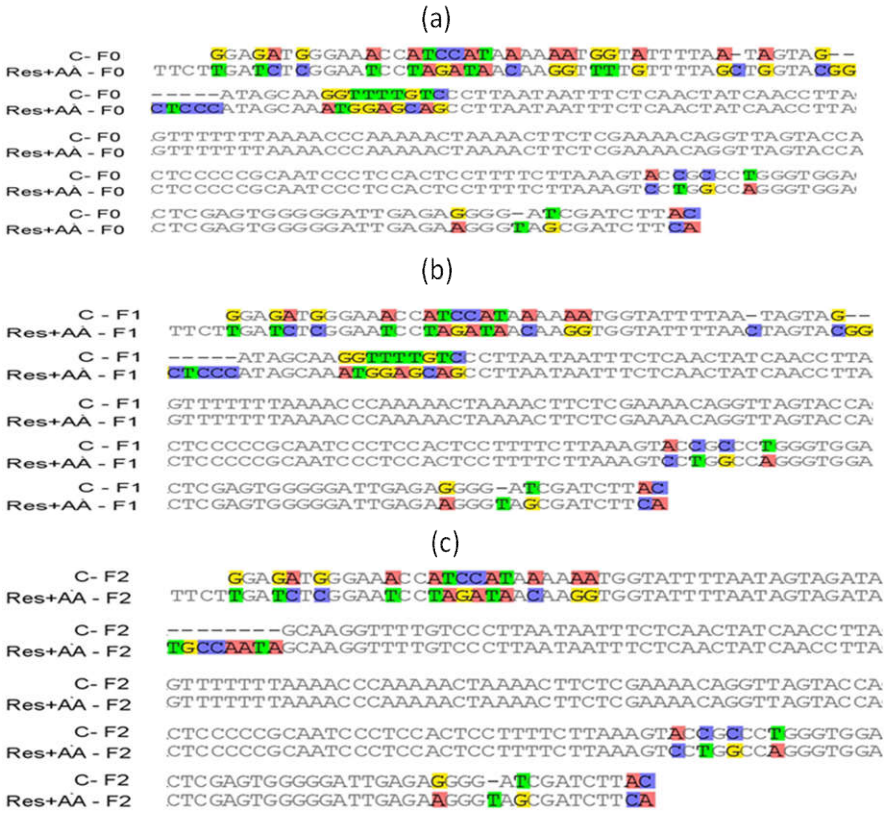

Fig. 7. Protective effect of resveratrol on the rate of mutations of *rpa-* 1 gene of nematode *C. elegans* generation

Table 1. Primers used for RT-qPCR

S.No.	Gene	Primers		Reference
		Forward	Reverse	
1	*cyp35a2*	5'-CTGGTGCTCTGGTCACTTCTCA-3'	5'-CCGAAGATCAAATACAATTCTGCT-3'	Shu-hua *et al. 2011*
2	*sod-3*	5'-CCAACCAGCGCTGAAATTCAATGG-3'	5'-GGAACCGAAGTCGCGCTTAATAGT-3'	Li *et al* 2008
3	*daf-2*	5'-CGGTGCGAAGAGAGGATATT-3'	5'-TACAGAGGTCGCCGTTACTG-3'	Li *et al* 2008
4	*daf-16*	5'-CCAGACGGAAGGCTTAAACT-3'	5'-ATTCGCATGAAACGAGAATG-3'	Li *et al* 2008
5	*ugt-44* (NM_069532.4)	5'-TCGGAACCAAGGTAGCCAAC-3'	5'-ACCAAGATGGGAATCGGTGG-3'	Designed in present study
6	*gst-2* (NM_069446.3)	5'-ACAGTCGGCTTCAATCGGAA-3'	5'-CATCACCAACCAGGAAACCG-3'	Designed in present study
7	*gst-4* (NM_069447.6)	5'-CTCTTGCTGAGCCAATCCGT-3'	5'-GCAGTTTTTCCAGCGAGTCC-3'	Designed in present study
8	sdz-8 (NM_073520.1)	5'-CTGCTGAGCTACGGAACGAA-3'	5'-TCTGTAGGCGAGAACTGGGA-3'	Designed in present study
9	msh-2 (NM_058801.5)	5'-GTGGAGGCTCTGGAGCTTTT-3'	5'-AGAAAGCCAATCACGGAGCA-3'	Designed in present study
10	*apn-1 (*NM_063286.5)	5'-AGACAATGGCTGGACAAGGG-3'	5'-TTCCATCGAGACGGTTGTCG-3'	Designed in present study
11	*exo-3 (*NM_001026414.3)	5'-CAAAACCCTCGTCGTCTCCA-3'	5'CGATTTGATTCTGGATTCCCCG-3'	Designed in present study
12	*xpa-1* (NM_059624.1)	5'-AAGCTTCGGTATTGGGCGAA-3'	5'TGCTGGCGGAGATCTTTGAG-3'	Designed in present study
13	*rpa-1* (NM_063205.4)	5'-GCAAATCCACCAGCAGCAAA-3'	5'-TTTCTGTGTTCGACGCGGTA-3'	Designed in present study
14	*ddb-1* (NM_069898.7)	5'-AACACGACCACCGAGTCTTC-3'	5'-ACAATCTCGATAGGCGCTGG-3'	Designed in present study

Table 2. DNA adduct of AA formed in gravid stage *C. elegans* exposed to different concentration AA

Concentration of AA	DNA adduct formed
0.0005 mg/L	N7-(2-carbamoyl hydroxyethyl)guanine, N7,9-bis-(2-carbomylethyl)guanine, N7-(2-carbamoyl hydroxy ethyl)guanine N6-(2-carboxyethyl)deoxy adenosine
0.005 mg/L	N7-(2-carbamoyl hydroxy ethyl)guanine, N7-(2-carbamoyl hydroxy ethyl)guanine, N1N6-(2-hydroxy propanoyl)deoxy adenosine, N6 .(2-carboxyethyl)deoxy adenosine
0.05 mg/L	N3-(2-carbomoylethyl)thymine,N7,9-bis-(2-carbomylethyl)guanine, N3-(2-carbomoyl ethyl)thymine, N6-(2-carboxyethyl)deoxy adenosine, N1-(2-carboxy ethyl)deoxy guonosine
0.5mg/L	N7,9-bis-(2-carbomylethyl)guanine, N3-(2-formamidoethyl) thymidine, N3-(2-carbomoyl ethyl)thymidine, N6-(2-carboxyethyl)deoxy adenosine
5 mg/L	N6-(2-carboxyethyl)deoxy adenosine, N7-(2-carbamoyl hydroxy ethyl)guanine, N7,9-bis-(2-carbomylethyl)guanine, N3-(2-formamidoethyl)thymidine, N3-(2-carbomoyl ethyl)thymidine,
50 mg/L	N6.(2-carboxyethyl)adenine, N7,9-bis-(2-carbomylethyl)guanine, N3-(2-formamidoethyl)thymidine, N3-(2-carbomoylethyl) thymidine, N6- (2-carboxyethyl)deoxy adenosine
500 mg/L	N7,9-bis-(2-carbomylethyl)guanine, N3-(2-formamidoethyl) thymidine, N1N6.(2-hydroxy propanoyl)deoxy adenosine, N6-(2-carboxyethyl)deoxy adenosine, N3-(2-carbomoyl ethyl) thymidine, N1(2-carboxy ethyl)deoxy guonosine, N3N4-(2-hydroxypropanoyl)-deoxycytidine

Table 3. DNA adduct formation at different stages of growth of *C. elegans*

DNA adduct formed in L1/L2 Stage of *C. elegans*	DNA adduct formed in L3/L4 Stage of *C. elegans*	DNA adduct formed in Gravid Stage of *C. elegans*
N7,9 –bis-(2-carbomylethyl) guanine	N7,9 bis-(2-carbomylethyl) guanine	N3-(2-carbomoylethyl) thymine
N6-(2-carboxyethyl)deoxy adenosine	N3-(2-formamidoethyl) thymidine	N3-(2-formamidoethyl) thymidine
N3-(2-formamidoethyl) thymidine	N3-(2-carbomoyl ethyl) thymidine	N3-(2-carbomoylethyl) thymidine
	N6-(2-carboxyethyl) deoxy adenosine	N6-(2-carboxyethyl)deoxy adenosine
		N1N6-(2-hydroxy propanoyl) deoxy adenosine
		N6- (2-carboxyethyl) adenine
		N3N4-(2-hydroxypropanoyl) deoxycytidine
		N1-(2-carboxyethyl)deoxy guonosine
		N7,9-bis-(2-carbomylethyl) guanine
		N7-(2-carbamoyl hydroxy ethyl)guanine

Table 4. Number and types of transition mutations in the *rpa*-1 gene of *C. elegans* generations exposed to AA

Transition mutation (number)			
Types of transition	**F0**	**F1**	**F2**
$A^a \rightarrow G^b$	22	27	20
$G^b \rightarrow A^a$	6	7	7
$T^d \rightarrow C^c$	17	23	18
$C^c \rightarrow T^d$	7	9	7

Table 5. Number and types of transversion mutations in the *rpa*-1 gene of *C. elegans* generations exposed to AA

Transversion mutation (Number)			
Types of transversion	**F0**	**F1**	**F2**
$G^b \rightarrow C^c$	2	2	1
$C^c \rightarrow G^b$	6	5	3
$A^a \rightarrow C^c$	10	10	10
$C^c \rightarrow A^a$	12	11	11
$T^d \rightarrow A^a$	9	9	8
$A^a \rightarrow T^d$	6	8	7
$T^d \rightarrow G^b$	12	12	11
$G^b \rightarrow T^d$	4	3	4

Table 6. Protection of resveratrol on mutagenicity caused by AA exposure of *rpa*-1 gene of *C. elegans* generations

C. elegans generations	AA (% identities)	Resveratrol +AA (% identities)
F0	41	78
F1	42	80
F2	41	85

Stress-related gene expression profiling analysis was conducted on the selected genes. We targeted few metabolic and DNA repair genes. Metabolic genes studied included xenobiotic metabolism enzyme genes such as cytochrome P450 (*cyp*-35A2), UDP- glycosyl transferases (*ugt*-44), short chain dehydrogenase gene (*sdz*-8), glutathione S transferases (*gst*-4), antioxidant enzyme genes namely superoxide dismutase (*sod*-3), and life span longevity genes (*daf*-2 and *daf*-16). The DNA repair genes studied were DNA mismatch repair protein **(***msh*-2), DNA-(apurinic or apyrimidinic site) lyase (*apn*-1), nematode larval development, base-excision repair and embryo development (*exo*-3), xeroderma pigmentosum (*xpa*-1), Replication protein A (*rpa*-1) and damage-specific DNA binding protein 1 (*ddb*-1). In the quantitative RT-PCR assay, AA caused an increase in the expression of *cyp*-35A2, *ugt*-44 genes compared to that of control. Expression of other metabolic genes, *gst*-4, *sdz*-8, *sod*-3, *daf*-2, and *daf*-16 were downregulated in AA-treated gravid stage worms. Resveratrol preexposure of worms for 24 h before AA exposure had a good protective effect on certain metabolic genes. *gst*-4, *ugt*-44 and *daf*-2 genes were upregulated, with the highest upregulation observed in *ugt*-44 followed by *gst*-4 and *daf*-2. The *daf*-2 gene was upregulated by only one fold compared to AA exposed worms (Fig 4a). Among the DNA repair genes studied, all genes were downregulated due to AA exposure. *Xpa*-1 was most effected followed by *apn*-1 gene. Resveratrol preexposure of worms for 24 h before AA exposure had a good protective effect on repair genes studied. All DNA repair genes studied were upregulated except *exo*-3. The upregulation was in the order of *xpa*-1 > *apn*-1 > *rpa*-1 > *msh*-2 > *ddb*-1 (Fig 4b).

Mutagenicity caused by AA in *C. elegans* generations

The sequence homology studied using Clustal W of the *rpa*-1 gene of different generations of *C. elegans* exposed to AA have shown mutation rate of 59% in F0, 58% in F1 and 59% in F2 generations. As expected, base substitutions were the most frequent mutations observed in all generations of *C. elegans* studied. Mutation rates were almost consistent across different replicates indicating that the accumulation of *de novo* mutations *per se* did not significantly change the

observed mutation rate, at least within the experimental period. The possible single-base substitutions occurred at broadly equal frequency between generations. Among transitions, A→G and T→C were most common with 8.56 and 6.61 % mutation of the *rpa*-1 sequence in F0 generation. In F1 and F2 generation the transitions, A→G and T→C were respectively 10.5 and 8.5 and in F2 generation, A→G and T→C were 8.95 and 7% respectively. With F0 to F1 generation, the repair of AA-induced damage in terms of transitions was not observed. However, F1-F2 generation there was 26% and 22% of repair of these bases (Fig 5, Table 4).

Transversion mutations of bases were also observed due to AA exposure. Transversion of A→C, C→A and T→G were high followed by T→A, A→T and C→G. C→A transversion was highest with a score of 4.67 % in F0 generation. However, in F1 and F2 generations transversion was 4.28 and 4.25 % respectively. Transversion of T→G was almost similar to C→A with a score of 4.67, 4.67 and 2.26 % in F0, F1, and F2 generations respectively. Transversion of A→C was 3.9% in all the three generations studied. T→A transversion was 3.5, 3.5 and 3.1 % in F0, F1 and F2 generations. Transversion of A→T and C→G were less with a score of 2.3, 3.1, 2.72 and 2.3, 1.95, 1.17 % respectively at F0, F1, and F2 generations. Transversions of G→C was lowest in all three generations studied. Thus, the total mutation rates were 59, 58 and 59 % respectively at F0, F1, and F2 generations (Fig 6, Table 5).

Protective effect of resveratrol on Mutagenicity caused by AA in *C. elegans* generations

Preexposure of worms with resveratrol for 24 h before AA exposure had protective effect on the mutations caused by AA in the *rpa*-1 gene of *C. elegans* generations studied. The sequence homology studied using Clustal W of *rpa*-1 *gene* of different generations of *C. elegans* preexposed to resveratrol have shown the increase in the sequence homology of 37%, 38% and 44% in F0, F1, and F2 generation respectively (Fig 7, Table 6), thus indicating protection from mutation caused by AA exposure.

DISCUSSION

We examined the effect of AA on the biological effect of continuous exposure to AA at levels that was found in common foodstuffs using *C. elegans* model in the present study. AA is formed in foods containing sugars and proteins at elevated temperatures by milliard reaction. AA was reported to be formed in concentrations of 30-2300 µg/kg during the frying or baking of many carbohydrate-rich foodstuffs (Tareke *et al*., 2002). As per the Swedish National Food Administration (SNFA, 2002), humans are being exposed continuously to several tens of micrograms of AA from their daily diet. In the present study, we examined the effects of AA on the growth, fecundity, lifespan, expression of few of the metabolic and DNA repair genes and study of mutations of the *rpa-1* gene, a DNA repair gene of the nematode *C. elegans*. *C. elegans* was chosen as an *in vivo* model because of its ease of handling, short lifespan, large brood size, easy availability of a large population for statistical analysis, amenable genetics and various other advantages. *C. elegans* has been proved to be a good model system to study the *in vivo* effects of chemical compounds and their operating mechanisms (Miwa *et al*., 1982; Tabuse *et al*., 1989). The maximum theoretical concentration of AA allowed in drinking water and foods by the WHO and US EPA is 1 mg/L (http://water.epa.gov/drink/contaminants/index.cfm#8). The reference dose established for AA by the EPA in 2010 was 0.002 milligrams of AA per kilogram of body weight per day .i.e. for a person weighing 150 pounds; this Reference Dose (RfD) for AA means a daily dietary exposure limit of about 140 µg. It has also estimated that an adult weighing 150 pounds averages about 27 micrograms of daily AA intake from his or her diet (USEPA 2010). However, the daily intake of AA by man is very much higher than this limit. Hence, we chose higher concentrations of AA in our studies.

In our *in vivo* trials, *C. elegans* was exposed to different concentrations of AA for the analysis of egg laying and brood size. The result showed a concentration-dependent decrease in the number of eggs laid and progeny. Also, AA caused a decrease in the lifespan of *C. elegans* and effected the worm locomotion by reducing the motility. AA affected the unexposed offspring of the exposed parental generation. In rats and mice injected intraperitoneally with 14C-radiolabeled AA, radiolabel was primarily associated with protamines of sperm in male germ cells (Segerback *et al*., 1995), and AA exposure reduced sperm mortality and fertility (Zenick *et al*., 1986). In the present investigation, the protective effect of the well-known exogenous antioxidant resveratrol was studied on on AA-induced toxicity in *C. elegans*. Resveratrol is reported to have high antioxidant activity and was believed to be a central factor in the French Paradox (Aschemann *et al*., 2015; Yamagata, 2015). Resveratrol can quench ROS such as superoxide and hydroxyl radicals. It especially protects the mitochondria, an organelle that produces high concentrations of ROS (Kitada, 2011). Resveratrol and two of its analog have been reported to exhibit a variety of potential biological effects such as anticancer effects (Xia *et al*., 2010), cardiovascular disease prevention (McCormack, 2013), and apoptosis induction (Jancinova, 2013). In this study, we observed the protection conferred by resveratrol on the toxicity of AA, by increasing growth, brood size, locomotion and extended the lifespan of the worm pre-exposed to resveratrol. Some toxicants, such as electrophilic chemical species, have the capacity to interact with nucleophilic sites and, thus modulate the structure of DNA molecules. This phenomenon might result in the formation of a DNA adduct which is a complex formed by the chemical interaction of a xenobiotic to DNA. The chemical carcinogens or their metabolites bind with DNA to exert their biological effects (Miller, 1970). Reactions of carcinogens or their metabolites with DNA are believed to be responsible for the mutagenic action and possibly the initiation of the malignant change of a wide spectrum of compounds. AA has been reported to bring DNA modification by the formation of DNA adducts. As DNA adducts would reflect a biologically significant dose, knowledge of the nature and amounts of DNA adducts formed *in vitro* / *in vivo* gives valuable information regarding the mutational effects that may result from particular exposures (Farmer *et al*., 2005). In our studies, more adducts were formed in the gravid stage. L1,/ L2, and L3/L4 stages also showed DNA adduct formation, but the

adducts were less in number compared to gravid. This study indicated that gravid stage was more susceptible than the other stages in the life cycle of the worm. In all the stages cytosine adducts were not observed. Probably the transversions of C to A and C to G were very high. Hence, more of adenine adducts were observed.

AA is a xenobiotic. The detoxification of xenobiotic is divided into two phases. CYPs and SDRs catalyze the reactions that make up phase 1 metabolism and allow for subsequent detoxification in phase 2. CYPs are the principal phase 1 enzymes and comprise a superfamily of heme-containing monoxygenases (Menzel *et al*., 2001). The short chain dehydrogenase/reductases which are found in the smooth endoplasmic reticulum and the cytosol catalyze the reduction of carbonyl groups in aldehydes and ketones. Phase 2 metabolism is the actual detoxification reactions of xenobiotic metabolism, comprising UGTs and GSTs. GSTs catalyze glutathione conjugation rendering nontoxic derivatives of xenobiotic compounds (Gibson *et al*., 2001). The transcriptional analysis by microarray showed that many genes were affected differently between 0.5 µg/L and 500 mg/L AA. Genes encoding glutathione-S-transferase (GST) exhibited increased expression levels in *C. elegans* exposed to 500 mg/L AA but did not increase the expression at 0.5µg/L (Hasegawa *et al*., 2008). GST has been known to act as a cellular detoxifier of both xenobiotic and endobiotic compounds (Salinas *et al*., 1999), including AA (Odland *et al*., 1994). Genes for a large number of major detoxification enzymes, such as GST, UGT, and SDR, were upregulated more than twofold by the AA exposure. Interestingly, AA upregulated the two other genes *gst*-2 and -3 closely located on the same chromosome as *gst-4* (Hasegawa *et al*., 2008). Overexpression of *gst*-4 showed resistance conferred on nematodes against oxidative stresses (Leiers *et al*., 2003). In the AA detoxification pathway of vertebrates, AA was metabolized by GST to mercapturic acid and excreted in urine. Whereas in the alternate pathway, AA was metabolized by CYP, a phase I enzyme, to GA, which may be mainly responsible for AA caused DNA damage (Friedman, 2003). Finding of many upregulated SDRs suggested that SDRs, not CYPs, are the primary phase I enzymes that are involved in AA metabolism in *C. elegans* (Hasegawa *et al*., 2008).

In our study, we selected *cyp*-35A2, *sdz*-8, *ugt*-44 and *gst*-4 to identify AA toxicity. *cyp*-35A2 (phase 1), *ugt*-44 (phase2) were upregulated and other metabolic genes, *sdz*-8 (phase 1) , *gst*-4 (phase2) was downregulated. *sod*-3, an oxidative stress-related gene was also downregulated indicating AA-induced toxicity of *C. elegans* was possibly related to stress-related xenobiotic metabolism genes. *gst*-4, *ugt*-44 genes were upregulated upon resveratrol pretreatment followed by AA, probably because of resveratrol protection. Decreased DAF-2 (insulin/IGF1) signaling extends *C. elegans'* life span up to threefold and causes increased fat storage and dauer arrest (Kimura *et al*. 1997). *daf*-16 is negatively regulated by the *daf*-2 pathway and has been the major downstream mediator of genes that extend lifespan (Lin *et al*., 1997; Ogg *et al*., 1997). The AA exposed *C. elegans* showed downregulation of *daf*-2 and *daf*-16 genes and upregulation of *daf*-2 gene upon resveratrol pretreatment in the present investigation. The genotoxic effect was observed to be heritable and was carried to further generations. *C. elegans* was used as an experimental model to study the effects on mutational signatures of DNA repair genes (*rpa*-1 gene), and their interactions with AA. AA-induced substitutions of guanines in GpC context. There were possible single-base substitutions that occurred at a broadly equal frequency between generations. These mutations were observed to be passed on to F1 and F2 generations. The total mutation rates were 59, 58 and 59 % respectively at F0, F1, and F2 generations. These types of base substitutions have been reported in aflatoxin exposure (Meier *et al*., 2014). Mutation is associated with developmental and hereditary disorders, aging and cancer. In a study using *C. elegans* by Meier *et al*. (Meier *et al*., 2014), exposure to carcinogens resulted in 1559 base substitutions, 406 indels, and 281 genomic rearrangements. From data, they extracted mutational signatures that resembled those seen in human cancers and developmental genomic disorders. The mutational burden increased with impaired nucleotide excision repair.

The expression of DNA repair genes studied showed downregulation of *msh*-2, *apn*-1, *exo*-3, *xpa*-1, *rpa*-1, *ddb*-1 and this may be the result of mutation caused by AA. Resveratrol, a naturally occurring polyphenol, exhibited protection on the genotoxic effect of AA (this study). Resveratrol was observed to protect against DNA damage induced by treatment with hydrogen peroxide (Livingston, 2015). To assess the ability of resveratrol to protect *C. elegans* from DNA damage, we pretreated *C. elegans* with resveratrol and then exposed them to AA. Resveratrol demonstrated the ability to protect the nematode from DNA damage. There was no loss of viability or increase in DNA damage after pretreatment with resveratrol in our study. So pretreatment may protect *C. elegans* to undergo cell death following oxidative stress. Prevention of DNA adduct formation indicated an ability of resveratrol to protect directly against DNA damage. Also, it could be that potentially resveratrol may protect changes in nucleosome positioning or chromatin structure from AA that result in decreased ability of AA to damage the DNA. The mechanisms for these results need to be further characterized. Also, resveratrol treatment protected the damage to the genes *gst-4*, *ugt-44* and *daf-2* and these genes were upregulated (this study). Monoadducts are known to be repaired by the base excision, nucleotide excision, and mismatch repair pathways (Meier *et al*., 2014). The upregulation of few repair genes studied in resveratrol-treated worms indicated the probability of DNA repair in the mutated genes (this study).

Conclusion

In conclusion, the advantages and usefulness of *C. elegans* as an organism of choice for toxicity (or safety) testing and other environmentally-related evaluations have been amply demonstrated by many researchers over the years. *C. elegans* genomes are used as a model to analyze the patterns and etiology of mutations arising with exposure to carcinogens and across different DNA repair backgrounds. The present investigation has demonstrated that resveratrol, one of the most abundant flavonoids in the human diet, is highly effective in reducing the DNA damage caused by the AA. Thus, the *in*

vivo findings using *C. elegans* model presented here suggest that resveratrol-containing diets may reduce carcinogen/ mutagen induced toxicities. The protective activities of resveratrol might promise its potential as chemo-preventive agent against the genotoxicity related with AA.

Acknowledgements

I acknowledge Indian Council of Medical research (ICMR) for providing the financial support and Director, CSIR- CFTRI for supporting the work.

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