



Ultra-Highly Diluted Homeopathic Remedy Arnica Montana 30C can Reduce UV-induced DNA Damage in *Escherichia coli* through its Regulatory Influence on Nucleotide Excision Repair Genes: A Commentary on our Published Research Finding



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Abstract

In homeopathy, ultra-highly diluted drugs are used with great benefits to treat various ailments in human and higher animals, but whether lower organisms (prokaryotes) with simple genetic system can also respond to homeopathic drugs was not known. To test if the homeopathic drug, Arnica Montana 30C (AM-30C), used against shock and injury, can modulate relevant gene expressions and oxidative stress, we exposed *Escherichia coli* (*E. coli*) to two doses of ultra-violet (UV) radiation and subsequently quantified its DNA damage and oxidative stress along with the expression of the nucleotide excision repair genes, *uvr A*, *uvr B* and *uvr C* in the drug-treated and placebo-treated (succussed alcohol, being “vehicle” of the drug) bacteria. Generation of reactive oxygen species (ROS) was also estimated and the gene expression patterns in them were compared. Certain other relevant parameters of study like comet assay, gel electrophoresis for DNA ladder, intra-cellular ROS generation, and other toxicity biomarkers like superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH) were also conducted. Expressions of mRNA of the excision repair genes estimated by reverse transcriptase polymerase chain reaction (RT-PCR) method were analyzed. AM-30C treated bacteria showed less DNA damage and diminished oxidative stress, along with decrease in ROS generation, and a corresponding increase in SOD, CAT and GSH activities. AM-30C up-regulated the expression of the repair genes *uvr A*, *B* and *C*, validating *prima facie* the “gene regulatory hypothesis” that claims ultra-highly diluted homeopathic drugs to act through regulation of relevant gene expressions as the most plausible molecular mechanism.

Keywords: UV Radiation; *Escherichia coli*; DNA Damage; Intracellular ROS; Arnica Montana 30C; Nucleotide Excision Repair

Abbreviations: ROS: Reactive Oxygen Species; NER: Nucleotide Excision Repair; UV: Ultra-Violet; CAM: Complementary and Alternative form of Medicines; CAT: Catalase; GSH: Glutathione; SOD: Superoxide Dismutase

Introduction

Ultra-violet (UV) radiation is an effective carcinogenic and mutagenic agent which is known to interact either directly with DNA or indirectly through generation of free radicals or reactive oxygen species (ROS), and can inflict DNA damage or alter the genomic integrity [1-4]. This can drastically affect the normal life processes of all organisms ranging from lower prokaryotes to higher eukaryotes [5-6]. For this reason, most living organisms have developed an intrinsic mechanism to repair their damaged DNA at the earliest. Cells have developed a number of repair or tolerance mechanisms to counter various forms of DNA damage and genotoxicity. The biochemical and molecular repair pathways have been extensively investigated in some model organisms such as *Escherichia coli*, *Saccharomyces cerevisiae*, and human [7], where specialized repair mechanisms are put in place. The repair

mechanism involves certain proteins which are recruited to scan the genome continuously and detect DNA lesions, if any.

On finding any lesion, several distinct intrinsic repair mechanisms, such as photo reactivation, nucleotide excision repair (NER) [8-9] and some other specialized forms of repair system such as SOS response are triggered, of which NER is very effective. NER pathway in *E. coli* has been extensively studied and is established to be primarily one of the major repair pathways to come into action in case of DNA damage due to UV or ionizing radiations [7,10]. Therefore, we became interested to examine if the potentized remedy diluted beyond Avogadro's limit (by a factor of 10^{60} in the study under present consideration) could have any role or could influence particularly on the activities or expression levels of these repair genes in *E. coli* that are known to regulate synthesis of the

UvrA, *B*, *C* endonuclease enzyme complex consisting of the *UvrA*, *B*, *C* proteins, and DNA helicase II (sometimes also known as *UvrD* in this complex), catalyzed by mainly three genes (*uvrA*, *B* and *C*). The protein *UvrA* recognizes the damaged DNA and recruits *UvrB* and *UvrC* to the site of the lesion. *UvrB* and *UvrC* then cleave on the 3' and 5' sides of the damaged site, respectively, thus excising an oligonucleotide consisting of 12 or 13 bases.

The action of helicase is then required to remove the damage-containing oligonucleotide from the double-stranded DNA molecule, and the resulting gap is filled by DNA polymerase I and sealed by ligase [7] subsequently. Arnica Montana 30C (AM-30C) is claimed in homeopathic literature to have a profound effect against internal shock and injury [11]. In earlier studies, wound-healing and anti-shock response properties as well as anticlastogenic action of potentized Arnica Montana have been reported in mice [12-14]. Similarly, report of AM-30C ameliorating genotoxic effects of ultrasonic sound waves in mice [15] is also on record. In recent years, people feel scared of unwanted and toxic side-effects of many modern medicines and are getting more inclined to take complementary and alternative form of medicines (CAM) because of their less or negligible side-effects. Among CAM, homeopathy is very popular, particularly because it often uses micro doses of very high dilutions of natural substances originating from plants, minerals or animal parts [16-17], and because of its no or negligible side-effects. However, its efficacy is challenged by rationalists because they think the ultra-highly diluted remedies used in homeopathy cannot act because of the ultra-high dilution of the original drug substance and also the molecular mechanism of action of such drugs is not unequivocally accepted. We therefore used AM-30C in the study to make sure that in this remedy, the dilution factor being by 10^{60} , existence of even a single molecule of the original drug substance in it becomes highly improbable.

Although some researchers demonstrated the existence of nanoparticles of the original drug substance in such ultra-highly diluted homeopathic drugs [18], the efficacy is often questioned by rationalists, as the precise mechanism of drug action is still scientifically unknown. Therefore, in our opinion, in-depth research is warranted to validate the efficacy of ultra-high dilutions in genetically simple unicellular living forms and try to understand the molecular mechanism of its action, if possible, in them. Thus, in our earlier study [19] on which the present commentary is based, we wanted to test the hypothesis if the potentized remedy AM-30C, used against shock and injury in human beings, could show its efficacy in reducing DNA damage caused by UV insult in *E. coli*, and if it could, to understand the possible molecular mechanism involved to accomplish it.

Materials and Methods

E. coli were maintained in the standard Luria-Bertani medium and UV-irradiated. In this study, two sub-lethal doses ($(25 \text{ J/m}^2$ and 50 J/m^2) were chosen from the range-finding trial and the dose-response curve. The UV-irradiated *E. coli* were divided into two groups:

- a) Those receiving AM-30C (drug treated) and
- b) Those receiving placebo (Alc. 30C-positive control).

Two groups of untreated *E. coli*, one receiving no drug and another receiving only AM-30C were also maintained to see if the drug itself had any adverse effects on *E. coli*. These latter set-ups also served as additional controls. The parameters, such as, estimation of intracellular reactive oxygen species (ROS) generation (by flow cytometric study), total thiol content (GSH by biochemical method), superoxide dismutase and catalase assays (by spectrophotometric study), comet assay (by fluorescence microscopy) for determining extent of DNA breakage, and m-RNA study, cDNA preparation and gene level expression study (by reverse transcription-polymerase chain reaction method) were conducted as per standard procedures [19] in a blinded manner and taking proper statistical methods into consideration.

Results

Generation of ROS was the minimum in the negative control groups. Much ROS was generated when cells were treated with UV-rays. Treatment of AM-30C decreased ROS generation in comparison to the only UV-treated groups as well as with UV-plus placebo-treated groups. The specific activity of SOD in *E. coli* cells was observed to have decreased after UV dose 1 and dose 2 treatments, in comparison with the corresponding controls. AM-30C treated cells showed significant increase in SOD specific activity while addition of placebo did not make any significant difference. In UV-irradiated groups, the specific activity of catalase in *E. coli* cells significantly decreased. Addition of AM-30C to both the UV doses 1- and 2-treated cells showed significant increase in catalase activity. Treatment with placebo did not make much difference in specific activity of catalase in *E. coli*. The concentration of the free GSH in *E. coli* cells significantly decreased after UV treatments. Administration of AM-30C showed considerable increase in the intracellular concentration of free glutathione as compared to only UV-treated groups while addition of placebo did not make any significant difference.

UV-treated groups showed a smeared or uniformly distributed damaged DNA (smear indicates extensive DNA fragmentation) with respect to UV un-irradiated control cells showing a clear single band, representing an intact DNA. AM-30C-treated groups also showed a clear single band, while the placebo-treated cells also showed DNA smearing like that of the only UV-treated *E. coli*. There was almost no DNA damage in UV un-irradiated cells. The comet tail lengths significantly increased in UV--treated groups (indicating more DNA damage) as compared to control. AM-30C treatment to UV-irradiated cells showed considerable decrease in comet tail lengths. The placebo-treated groups also showed considerable increase in comet tail length like that of the UV-treated groups. Thus the experimental values indicated that the drug reduced the quantum of DNA damage induced by UV radiation. The analysis of mRNA expression levels of *uvrA*, *uvrB* and *uvrC* genes and house-keeping gene (glyceraldehyde-3-phosphate dehydrogenase) of *E. coli* cells in different control and treatment groups indicated that

the arbitrary band intensity level of *uvrA*, *uvrB* and *uvrC* genes showed increased m-RNA expression levels both in UV treated cells when compared to UV un-irradiated control cells, whereas, in case of AM-30C-treated groups, the arbitrary band intensity resulted in a further significant increase in m-RNA expression levels of *uvrA*, *B* and *C* genes. Addition of placebo did not make much significant difference in band intensity from that of only UV treated groups.

Discussion

Results of the present study revealed DNA damage and generation of oxidative stress in *E. coli* as a result of exposure to UV radiation, more in quantity at the longer and higher exposure. The consequences of UV-irradiation included generation of ROS, DNA damage, and decrease in levels of SOD, CAT and GSH. Interestingly, after exposure to UV radiation, the expression of *uvrA*, *uvrB* and *uvrC* genes responsible for repair of DNA damage showed an up-regulation, as compared to UV un-irradiated control. It is known that UV radiation induces damage through the generation of free radicals or reactive oxygen species (ROS) [4-5]. The highly toxic superoxide anion (O_2^-) seriously disrupts normal metabolism through oxidative damage to cellular components. Oxygen free radicals can also be converted to reactive hydroxyl radicals, which again can cause DNA damage. Intracellular ROS attack both the bases and the sugar moieties, producing single and double-strand breaks in the backbone, adducts of base, sugar groups and cross-links to other molecules that block replication [20-21].

Therefore, elimination of superoxide anion is definitely necessary for survival of cells [22]. AM-30C significantly reduced intracellular ROS generation and increased activities of some biomarkers like SOD, CAT and free intracellular glutathione (GSH) content found to be decreased after UV exposure. SOD and catalase are the weapons to restrict the accumulation of reactive oxygen species. In our findings, both SOD and CAT showed marked increases in their specific activities on AM-30C treatment. In the UV-exposed *E. coli* administered AM-30C, there was less amount of DNA damage as determined from the analysis of data through Comet assay and DNA gel electrophoresis. Overall, AM-30C had an ameliorative effect on UV-exposed *E. coli*, which was not really due to "ethanol effect" as the placebo treated *E. coli* did not show such ameliorative responses. As DNA repair in *E. coli* is quite established scientifically to be accomplished through the activities of the specific repair genes, the over-expression of these genes was significant, depicting an up regulation and enhanced activities of these repair genes. Since the over-expression of these genes could not be found in the *E. coli* treated with the placebo, it could be logically concluded that AM-30C must have played the key role in triggering alteration in the expression level of these repair genes.

The results through RT-PCR also confirmed that mRNAs of *uvrA*, *uvrB* and *uvrC* genes were over-expressed. Therefore, the results of the present investigation actually vindicated the hypothesis first advocated by Khuda-Bukhsh [23-26] that one major way by which the potentized homeopathic drug would act is by the regulation of expression of the relevant genes. Khuda-Bukhsh and

his collaborators [27-29] also found many evidences of alteration in gene expression in a mammalian model (*Mus musculus*) after administration of various forms of homeopathic drugs and demonstrated change in many signal proteins in experimental cancerous mice *in vivo* and also in human cancer cells *in vitro* [30-36]. All these experimental results also would bear testimony towards validation of the strong arguments in favour of the "gene regulatory hypothesis" that explains the molecular mechanism of action of the potentized homeopathic drugs by their ability to trigger selective and relevant gene expressions as revealed also in the lower primitive form of unicellular organisms like *E. coli* with a simple genetic system.

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