Research Article

Homeopathic Thuja 30C ameliorates benzo(a)pyrene-induced DNA damage, stress and viability of perfused lung cells of mice in vitro

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OBJECTIVE: To examine if the ultra-highly diluted homeopathic remedy Thuja 30C can ameliorate benzo(a)pyrene (BaP)-induced DNA damage, stress and viability of perfused lung cells of Swiss albino mice in vitro.

METHODS: Perfused normal lung cells from mice were cultured in 5% Roswell Park Memorial Institute medium and exposed to BaP, a potent carcinogen, at the half maximal inhibitory concentration dose (2.2 µmol/L) for 24 h. Thereafter, the intoxicated cells were either treated with Thuja 30C (used against tumor or cancer) or its vehicle media, succussed alcohol 30C. Relevant parameters of study involving reactive oxygen species (ROS) accumulation, total glutathione (GSH) content, and generations of heat shock protein (hsp)-90 were measured; the cell viability and other test parameters were measured after treatment with either Thuja 30C or its vehicle media. Circular dichroism spectroscopy was performed to examine if Thuja 30C directly interacted with calf thymus DNA as target. For ascertaining if DNA damaged by BaP could be partially repaired and restituted by the remedy, 4’,6-diamidino-2-phenylindole staining was performed.

RESULTS:Thuja 30C increased cell viability of BaP-intoxicated cells significantly, as compared to drug-untreated or drug-vehicle control. A minimal dose of Thuja 30C significantly inhibited BaP-induced stress level, by down-regulating ROS and hsp-90, and increasing GSH content. Thuja 30C itself had no DNA-damaging effect, and no direct drug-DNA interaction. However, it showed quite striking ability to repair DNA damage caused by BaP.

CONCLUSION:Thuja 30C ameliorates BaP-induced toxicity, stress and DNA damage in perfused lung cells of mice and it apparently has no effect on normal lung cells.

KEYWORDS: Thuja 30C; benzo(a)pyrene; reactive oxygen species; oxidative stress; HSP90 heat-shock proteins; in vitro

DOI: 10.3736/jintegrmed2013054

1 Introduction

Homeopathy, a popular branch of complementary and alternative therapy, uses ultra-highly diluted natural substances originating from plants, minerals or animals. Homeopathic remedies are claimed to cure several diseases around the world. In homeopathy, drugs are used in the form of dynamized preparations obtained from serial dilutions and repeated agitations. Thus, when the drug attains the potency 12C, it becomes diluted to 10^-24, which is beyond the Avogadro’s limit. Such dilution beyond Avogadro’s limit raises the controversy about the lack of physical existence of any drug molecule, and therefore, its
efficacy is questioned. However, several studies have tried to define the efficacy of such highly potentized homeopathic remedies in biological systems[2,3].

Thuja occidentalis is a major plant largely produced in part of Europe. Earlier reports suggested that Thuja occidentalis mother tincture and its potentized forms are effective in treating several diseases like lung cancer, breast cancer, etc[4-7]. Thuja is reported to be an attractive drug for treating skin diseases including lesion and is also effective against diarrhea[8,9]. Earlier, Frenkel et al[9] and Banerji et al[9] claimed highly potentized form of Thuja such as Thuja 30C as an effective remedy against several diseases. In our previous study, we have shown that Thuja occidentalis extract, used as a homeopathic mother tincture, was effective against non-small cell lung cancer cell line, A549[10]. For further evaluation, we have tested the efficacy of potentized form of the same against lung cancer cell lines, A549 and H460. However, when used directly on the lung cancer cells, Thuja 30C was found to be less effective in killing these cancer cells at lower doses. Such results motivated us to test if the remedy plays any protective role against toxicity induced in normal perfused lung cells exposed to a major carcinogen, benzo(a)pyrene (BaP). BaP is one of the major lung carcinogens and constituents of cigarette, coal tar, etc[11,12]. It has previously been reported that it exerts its cytotoxic effect via deregulation of stress balance and thereafter damaging cellular DNA[11-15], which in turn enhances cellular mortality.

Therefore, our main objectives of the study were to check whether treatment of highly diluted homeopathic remedy Thuja 30C could be effective in protecting BaP-intoxicated lung cells to give them better survivability, to check if Thuja 30C ameliorates BaP toxicity in lung cells through regulatory control over stress responses, and to know if Thuja 30C has any demonstrable role in combating BaP-induced cellular DNA damage through a retrieval process.

2 Materials and methods

2.1 Reagents and drugs

Roswell Park Memorial Institute (RPMI)-1640 medium, fetal bovine serum (FBS), and penicillin-streptomycin-neomycin (PSN) antibiotic were purchased from Gibco BRL (Grand Island, NY, USA). Tissue culture plastic wares were obtained from Tarsons (USA). All organic solvents used were of high-performance liquid chromatography grade. 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 4',6-diamidino-2-phenylindole (DAPI), 2',7'-dichlorofluorescein diacetate (H$_2$DCFDA), calf thymus DNA, BaP, secondary antibodies, etc. were purchased from Sigma Aldrich (USA). Anti-heat shock protein (hsp)-90 monoclonal antibody was purchased from Cell Signaling Technology (USA). Ultra-high dilution of Thuja 30C and potentized alcohol (vehicle media of Thuja 30C) were obtained from Boiron Laboratory, France.

2.2 Animals

We housed a large group of healthy inbred strain of Swiss albino mice (6-8 weeks; body weight 20-25 g) for at least 14 d in an environmentally controlled room (temperature (24 ± 20) °C, humidity 55% ± 5%, 12-h light/dark cycle) with access to food and water ad libitum. The experiments were conducted under supervision of the Animal Welfare Committee of the Department of Zoology, University of Kalyani, India, and as per the approved ethical committee guidelines of the Institutional Ethical Committee, University of Kalyani, India (vide-892/OC/05/CPCSEA).

2.3 Lung cell perfusion

A total of 48 healthy Swiss albino mice were randomized and selected for the experiment. Mice were chloroformed before sacrifice. Mice lungs were dissected out and washed with Hank’s buffered salt solution with 0.1% collagenase. Then the tissues were minced into small pieces within 5% RPMI media and the lung cells were flushed out gently in the media with the aid of hypodermic syringe. Then the cells were passed through a thin nylon mesh (0.22 µm) and homogeneous single lung cell population was obtained. Then the cells were spun down at 1 000 × g for 3-4 min. The blood cells were pelleted out and the supernatant containing lung cells was taken. Viable lung cells were counted in haemocytometer by trypan blue exclusion test and used for further analysis[16].

2.4 Treatment of BaP and drug dose selection

For MTT assay, BaP at the concentration of 2.2 µmol/L, its half maximal inhibitory concentration (IC$_{50}$), was intoxicated at 37 °C in RPMI supplemented with 5% FBS. As positive control, equal volume of medium was added to the untreated cells[17].

For dose-dependent study, BaP-intoxicated cells were simultaneously treated with Thuja 30C for 24 h at the dose of 1-7 µL (for 100 µL culture media). Minimum dose (3 µL in 100 µL solvent media) that started showing an increase in cell viability was selected and used for subsequent experiments. The experiment was performed in triplicate, where each group was six in number.

2.5 Cell viability assay

Cell viability was determined using MTT assay. Briefly, perfused lung cells were seeded in 96-well plates at a density of 1×10$^4$ cells per well. They were allowed to settle for 24 h before treatment. Thereafter, the cells were intoxicated with BaP (2.2 µmol/L) with 5% CO$_2$ at 37 °C. Then the cells were simultaneously treated with Thuja 30C in an increasing concentration for 24 h. Cell viability was measured by MTT assay[18]. The control values corresponding with the untreated cells were taken as 100% and the viability data were expressed as percentage of control.

To observe whether Thuja 30C has cytotoxic effects on
normal lung cells, we performed MTT assay after treating the cells with only Thuja 30C at the highest concentration.

We also treated the BaP-intoxicated lung cells with the drug vehicle media to evaluate only the drug’s action, if any. The experiment was performed in triplicate, where each group was six in number.

2.6 Intracellular reactive oxygen species accumulation assay

Levels of reactive oxygen species (ROS) generation in cells were assessed fluorometrically using H₂DCFDA molecular probe. Cells of 1 × 10⁴ were intoxicated with BaP with simultaneous treatment of Thuja 30C (3 µL for 100 µL dose) for 1 to 6 h or left untreated. After incubation with 10 µmol/L H₂DCFDA for 30 min at 37 ºC in the dark, fluorescence was measured at excitation and emission wavelengths of the oxidized form at 504 and 529 nm, respectively[19]. Hydrogen peroxide (H₂O₂)-treated cells were taken as loading control. The experiment was performed in triplicate, where each group was six in number.

2.7 Protein isolation

Cells (5×10⁴) were plated in a 90-mm culture dish and were allowed to grow for 24 h. Following BaP intoxication and simultaneous treatment with Thuja 30C, cells were collected and lysed in 20 µL of ice-cold lysis buffer (10 mmol/L Tris-HCl, pH 7.5, 1 mmol/L MgCl₂, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.1 mmol/L phenylmethylsulfonyl fluoride, 5 mmol/L β-mercaptoethanol, 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate and 10% glycerol). Cells were incubated for 30 min on ice and centrifuged for 30 min at 5 000 × g at 4 ºC. After centrifugation the supernatant was collected and stored in −20 ºC for further use.

2.8 Glutathione estimation

Briefly, drug-treated (3, 6, 9 and 12 h) and untreated cells (1 × 10⁴) were taken and the cell extracts were prepared. We undertook spectrophotometric analysis to determine glutathione (GSH) content according to a method of Tietze[20]. The experiment was performed in triplicate, where each group was six in number.

2.9 Hsp-90 activity assay by indirect enzyme-linked immunosorbent assay

Totally 1 × 10⁴ cells were seeded in a 90-mm petridish and allowed to adhere for 24 h. Then the cells were intoxicated with BaP and simultaneously treated with Thuja 30C for 3, 6, 9 and 12 h. After respective time interval of drug exposure, protein was isolated. Thereafter, indirect enzyme-linked immunosorbent assay (ELISA) of hsp-90 protein was done according to the manufacturer’s protocol (Santa Cruz Biotechnology, Inc., USA) and quantified using an ELISA reader (Thermo Scientific, USA). We used para-nitrophenyl phosphate (pNPP) as a color-developing agent and measured the color intensity at 405-nm wavelength. The experiment was performed in triplicate, where each group was six in number.

2.10 Circular dichroism spectral analysis

Circular dichroism (CD) analysis was done by taking calf thymus DNA as a standard control. The analysis was done to explain whether Thuja 30C was capable of damaging DNA by interacting with it or not. The calf thymus DNA (ctDNA) concentration in the experiments was 100 µmol/L. CD spectra were recorded on a JASCO J720 CD spectrometer at 37 ºC using 1 mm cuvette[21]. The spectral recording was performed from 200 to 500 nm, repeated thrice and averaged automatically.

Induced CD spectral reading was performed resulting from the interaction of the Thuja 30C or its vehicle media and ctDNA at 37 ºC, which was obtained by subtracting the CD spectrum of the native DNA and mixture of both ctDNA and Thuja 30C or its vehicle media from the CD spectrum of the buffer and spectra of both buffer and Thuja 30C or its vehicle media solutions, respectively.

2.11 DNA damage assay by DAPI staining

Cells (2×10⁴) were plated in 6-well culture plates and allowed to grow for 24 h. Then cells were intoxicated with BaP and simultaneously treated with Thuja 30C for 3, 6, 9 and 12 h, respectively. After staining the cells with DAPI (10 µmol/L) for 15 min, fluorescence of DAPI was observed under a fluorescence microscope (Leica, Germany). The experiment was performed in triplicate, where each group was six in number.

2.12 Blinding

The observers were “blinded” during observations as to whether they were observing the “control” or “treated” materials.

2.13 Statistical analysis

All the experiments were performed in triplicate, where each group was six in number. Results were expressed as mean ± standard deviation, unless otherwise stated. Statistical analysis was made by one-way analysis of variance with LSD post-hoc test using SPSS 16.0 software. P<0.05 was considered statistically significant.

3 Results

3.1 Effects of Thuja 30C on cell viability

Normal mice lung cells incubated with 2.2 µmol/L of BaP for 24 h showed almost up to 50% of cell mortality as compared to the untreated control. But when these BaP-intoxicated cells were simultaneously treated with Thuja 30C (1-7 µL for 100 µL culture media), the cell viability was increased to 99.53% ± 0.59% at the dose of 3 µL (for 100 µL culture media) (Figure 1). However, at the dose range of 1-2 µL (for 100 µL culture media) the cell viability was 93.31% ± 0.49% and 93.04% ± 0.80%, respectively, but the differences in the percentage of viability as compared to BaP-intoxicated control were still at a statistically significant level.

Cells treated with drugs did not show any cellular cytotoxicity and reduction in viability. This however indicated the
drug’s ability of being effective in adverse cellular condition, not in any normal circumstances. Though after treatment with the vehicle of the drug, viability of BaP-intoxicated cells was increased to 86.49% ± 0.69%, the viability was still much less than the lowest Thuja 30C dose (1 µL for 100 µL culture media)-treated set where the viability was 93.31% ± 0.49%. Therefore, this result demonstrated greater effectiveness of the homeopathic remedy in BaP-intoxicated cells, as compared to only vehicle-treated cells.

3.2 Effects on cellular ROS generation

Fluorimetric results indicated that cellular ROS level got increased after BaP intoxication at an early time (3-12 h). However, cellular ROS level got down-regulated when those cells were simultaneously treated with Thuja 30C (3 µL per 100 µL culture media). At 3-6 h of exposure, Thuja 30C was unable to decrease the ROS level, but later at 9-12 h of exposure, BaP-induced elevated level of ROS was down-regulated (Figure 2), apparently looking like a biphasic effect.

3.3 Effects on GSH level

Enzymatic estimation revealed that intracellular GSH level was down-regulated after 3-12 h of BaP intoxication. However, simultaneous treatment of Thuja 30C (3 µL per 100 µL culture media) for 3-12 h significantly up-regulated cellular GSH level (Figure 3).

3.4 Effects on hsp-90 level

Indirect ELISA assay showed that hsp-90 got up-regulated after BaP intoxication, which presumably helped in controlling the stress level. Simultaneous treatment of Thuja 30C, however, helped in down-regulating hsp-90 level at 3-12 h (up to 62.17% ± 2.06% after 12 h), suggesting some reduction in stress level (Figure 4).

3.5 Drug-DNA interaction

CD spectral analysis results indicated that Thuja 30C apparently failed to interact with calf thymus DNA, so did the drug vehicle media (Figure 5). The CD spectra were observed to remain unchanged upon either drug or its vehicle media treatment; this would indicate that neither drug nor its vehicle media could trigger any conformational change of the DNA through direct interaction or had any damaging effect on DNA by itself.

3.6 Effects on DNA damage

DAPI staining revealed that BaP-intoxicated cells showed brighter fluorescence which indicated nicked and damaged DNA. It is interesting to note, however, that Thuja 30C-treated cells showed less fluorescence, indicative of the ability of the drug to reduce BaP-induced DNA damage (Figure 6).

Figure 1: Effects of Thuja 30C on the viability of BaP-intoxicated mice lung cells
Cells were either treated with only Thuja 30C (7 µL per 100 µL culture media) or exposed to 2.2 µmol/L BaP. BaP-exposed cells were simultaneously treated with drug vehicle media (7 µL per 100 µL culture media) and Thuja 30C (1 µL per 100 µL to 7 µL per 100 µL culture media). After 24 h of treatment cell viability was determined by MTT assay. Data are expressed as percentage of viability and each point expressed as mean ± standard deviation (n = 6). **P < 0.01, vs untreated group.
BaP: benzo(a)pyrene; MTT: 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide.
Discussion

Results of MTT assay indicated that cell viability decreased almost up to 50% when the normal lung cells of mice were intoxicated with BaP at a dose of 2.2 µmol/L. However, the cell viability of the intoxicated cells was found to be increased significantly in a dose-dependent manner when they were simultaneously treated with Thuja 30C, particularly after administration of 3 µL (in 100 µL culture media) or a higher dose of Thuja 30C. On the contrary, there was no significant change in the percentage of cell viability when the BaP-intoxicated cells were treated with drug vehicle media. However, the treatment of normal lung cells with Thuja 30C did not show any cytotoxic effect, which
implicates the potentiality of Thuja 30C to have a regulatory effect only when the cells were under the carcinogen-induced stress.

An efficient regulatory balance on oxidative stress helps a cell to survive in an adverse condition[19]. Thus the key regulatory act could possibly have been mediated through the regulatory control over the oxidative stress generated by BaP at certain stages to reduce the cytotoxicity induced by the carcinogen. This led us to make a more elaborative study on stress generation pathway and its possible modulation by the homeopathic remedy. The data on ROS generation, GSH content and hsp-90 activity also supported this regulatory process. Earlier, Kumar *et al*[14] reported that BaP mediates its toxicity by deregulating cellular redox state balance, particularly by generating ROS and down-regulating major antioxidants like GSH. From the results of this study, it seems that BaP at early hours modulated redox potential of normal lung cells by up-regulating the level of ROS and down-regulating the level of antioxidant, GSH. However, treatment of Thuja 30C at a minimal dose (3 µL per 100 µL culture media) was able to decrease ROS level from 9 to 12 h and increase GSH level from 3 to 12 h. At the earlier phases (3 to 6 h) of drug exposure alteration in oxidative stress response was dependent on GSH up-regulation rather than ROS depletion. However, from 9 to 12 h of drug exposure the oxidative stress alteration was carried out both by up-regulation of GSH and depletion of ROS. This would bear testimony to the ability of the potentized homeopathic remedy to normalize the stress condition generated by the BaP. The apparent bi-phasic effect on ROS generation has a similarity with certain effects mentioned as “hormetic effects” by some workers[22]. However, the hypothesis that equated “hormesis” as the cause of homeopathic effect is supported by some[22], but not by others[23]. In fact, the debate is still on and remains inconclusive, because hormesis is more of a phenomenon of favorable responses elicited by low dose toxin, but it does not really account for the precise molecular mechanism that is involved and it does not also clearly explain the homeopathic doctrines.

Hsp-90 is a part of molecular chaperones family that is responsible for protein folding and activation[24]. Hsp-90 is also involved in regulating protein degradation. In many a case of chemical toxicity hsp-90 level is up-regulated[25,26]. This elevation is attributed to an increase in cellular stress. In this study, we also observed an elevated level of hsp-90 after BaP intoxication. This may be the primary reason of cellular stress developed by BaP that in turn generated cytotoxicity. However, treatment of Thuja 30C reduced cellular level of hsp-90 in carcinogen-intoxicated cells as indicated by ELISA results. At 3-12 h of Thuja 30C exposure BaP-induced elevated level of hsp-90 got down-regulated. Thus, the other effective way to ameliorate stress could also be its ability to reduce hsp-90 generation by BaP that could render adequate protection to the cells towards their increased survivability.

Increased level of stress is also known to cause nuclear DNA damage[27]. In this study, increased level of DAPI fluorescence was observed after BaP intoxication which indicates cellular DNA damage. However, after simultaneous treatment with Thuja 30C, fluorescence level of DAPI was lowered. This suggests that DNA damage induced by BaP was partially recovered by Thuja 30C treatment. It is reported that some chemicals induce DNA damage itself by interacting with DNA[28]. However, if any agent (drug) bears the capacity to interact with DNA then it might be able to make some conformation changes in the DNA. In our present findings, CD spectral analysis revealed that Thuja 30C was unable to interact with calf thymus DNA. As the drug was not able to interact with calf thymus DNA in a cell-free system, therefore it would not presumably be able to interact with cellular DNA. Therefore, it may be extrapolated that this drug does not bear the self-reactive ability to damage DNA by interaction, rather bear the capability to reduce BaP-induced DNA damage. So the protection was apparently not mediated through direct conformational change in DNA. An overall scheme of important regulatory events that presumably occurred to render protection to intoxicated lung cells has been summarized in Figure 7.
Thus, Thuja 30C, an ultra-highly diluted remedy above Avogadro’s limit, showed quite convincing evidence of anti-carcinogenic effect in lung cells, possibly through its action at the molecular level of gene regulation as proposed by Khuda-Bukhsh[1,29] earlier, presumably because of the reported presence of nanoparticles of the drugs even in their highly diluted forms[30-32]. Further, amelioration of BaP-induced lung cell damage would signify this potentized remedy as a major putative agent in combating lung toxicity, which can be used as a supportive remedy in lung cancer.

5 Acknowledgements

Grateful acknowledgements are made to Boiron Laboratories, Lyon, France for the financial support of the work. This work was financially supported by a grant sanctioned to Prof. A.R. Khuda-Bukhsh, Department of Zoology, University of Kalyani, India, by Boiron Laboratories, Lyon, France.

6 Conflict of interests

The authors declare that they have no competing interests.

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