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ORIGINAL PAPER

Mice as a model for homeopathy research

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Mice (*Mus musculus*) have been used as a model for homeopathy research in relation to cytotoxicity, genotoxicity and carcinogenesis in our laboratory for the last three decades. Initially, anti-radiation activities of several potentized homeopathic drugs were tested against suitable controls by taking into consideration several cytogenetic endpoints. Subsequently, anti-cytotoxic, anti-genotoxic and anti-oxidative stress effects of some homeopathic drugs were tested against several chemical toxic metalloids and metal compounds. Modern techniques including Western blot, immunofluorescence, electron microscopy, UV-spectroscopy, HPLC, FTIR, NMR, RT-PCR etc were deployed to understand the possible mechanisms and pathways of action of potentized homeopathic drugs. We hypothesise that one way by which potentized homeopathic drugs act is through regulatory action on gene expression. *Homeopathy* (2009) **98**, 267–279.

Keywords: Homeopathy; Mice; Potentization; Techniques; Mechanism; Pathways

Introduction

An animal model is a non-human animal of disease or injury similar to a human condition. The disease or injury is often inflicted to test the efficacy of any drug that may have potential for treating the condition. Animal models of disease can be naturally occurring or induced by physical, chemical or biological means. For example the use of ionizing radiation to cause cytotoxic or genotoxic effect or tumors, or chemical carcinogens or other toxic agents to generate cancer in a specific organ and to test the efficacy of a proposed treatment.

Mice (*Mus musculus*) are often chosen as experimental animals in biology as they share a high degree of homology with humans, with 85% genomic similarities.[1] They have 20 pairs of rod like (acrocentric) chromosomes in metaphase; therefore, any numerical or structural changes in chromosomes can easily be detected. In male mice, spermatogenesis continues all year, facilitating evaluation of effect on sperm head morphology. The main objective of our studies was to test the efficacy of homeopathic drugs, particularly those diluted beyond Avogadro's limit, in mice. Mice were treated with mutagens, carcinogens or toxic chemicals to produce effects, cytotoxic, genotoxic or histo-pathological or patho-physiological in nature, then examined for possible modulatory effects as a result of administration of homeopathic drugs. The homeopathic drugs were generally chosen on the basis of their claimed effect on certain disease conditions. For example, *Arnica montana* is a homeopathic remedy used against shock and injury. Likewise, if mice are treated with a toxic agent or carcinogen acting on the liver, and if a homeopathic remedy having a corrective action on liver is chosen, it should give positive result, if it acts, showing amelioration of liver dysfunction, as compared to control animal that receives carcinogen, but only 'succussed alcohol' control. On the other hand, if a wrong homeopathic remedy which is not reported to have ameliorative effects on the particular organ is selected, a negative result would generally be predicted. Succussed alcohol control is a universal control for homeopathic remedies, having no initial drug substance before the 'potentization/dynamization' procedure, and therefore no so-called 'molecular imprints' of any particular initial drug substance. Thus the selection of the homeopathic remedy is important. 'Positive' results do not imply that all animals will necessarily show equal and same degree of improvement or amelioration. Positive results mean that the treated mice would show significant differences (in statistical term) when compared with

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controls. Thus when the results are mentioned as positive, it means that individual parameters differed significantly ($p < 0.05$ to $p < 0.001$) from controls. This would mean that not all mice receiving active treatment responded equally well and showed positive results in absolute terms, but nevertheless there was significant protection associated with the homeopathic remedy. However, when individual parameters of study, including cytogenetical endpoints, or biochemical parameters like alanine aminotransferase (ALT), aspartate aminotransferase (AST) etc. were considered, mice of the treatment group that showed the presence of a few liver tumors, might also show toxicity level as determined from biomarkers (enzymes) significantly less than that of control animals also showing tumors. Specific, quantifiable and meaningful parameters were chosen for assay before and after treatment. Protocols were selected which give some clue as to the possible mechanism of action of the homeopathic medicine, or the pathways had previously been firmly established. As for example, it is well known that if an animal is exposed to ionizing radiation, there will be damage to the chromosomes and DNA, and this can be quantified as well as qualitatively assessed. How radiation causes damage to the chromosomes and other cytogenetic endpoints and how they are repaired by natural processes is well established.[1,2] If a homeopathic medicine is found to have positive action and modulates well accepted endpoints, compared to appropriate controls, then one has a clue as to the probable mechanism of action as well. Thus the ultimate objective was to understand the overall *modus operandi* of homeopathic medicines, particularly those diluted beyond Avogadro's limit.

Methods

In all experiments, the emphasis was on maintaining suitable control(s) and ensuring repeatability of results. In our initial studies[3-5] we used several controls. As for example, against a potentized homeopathic medicine, one control group was treated with succussed alcohol, another with diluted alcohol (unsuccussed), another with only water, and another received no treatment at all. On critical examination, it was found that there were no statistically significant differences among the data scored for these controls. For example, in one such experiment, the chromosome aberration data of mice subjected to whole-body X-irradiation with 100 rad revealed total frequencies of aberrations in succussed alcohol treated, diluted alcohol treated, distilled water treated and 'no water' group mice to be 16.87 ± 0.1, 17.06 ± 0.1, 17.0 ± 0.1 and 16.93 ± 0.1, respectively, compared to 12.4 ± 0.3 in the *Arnica montana* 30C treated mice at 24 h.[4] In later experiments, to minimize the cost and lives of mice, we only maintained control mice treated succussed alcohol (say alcohol 30C as control against homeopathic medicine 30C, and alcohol 200C against homeopathic medicine 200C). On further critical examination on a large group, we found that there was hardly any difference found between 30C or 200C alcohol treated mice. Mice that were treated only with succussed alcohol or diluted alcohol also showed insignificant differences between them in all parameters of study. Therefore, in our recent studies we preferred to omit these controls retaining only one, succussed alcohol.

Blinding

The observers were 'blinded' during observation. Details of how this was accomplished has been described elsewhere.[6,7] Uniformity was maintained in scoring data of both control and treated animals.

Culture and maintenance of colonies

In laboratory experiments, it is necessary that the mice are homogeneous in their genome. Therefore, colonies of inbred Swiss albino mice were developed on a standard diet and water *ad libitum*. The hygiene of the colony was maintained as per the guidelines of the Institutional Animal Welfare Committee and all experiments were prior-approved by the Animal Ethical Committee, University of Kalyani.

Protocols used and their implications

We used standard protocols for every parameter of study, details of these can be obtained from our published papers.[3,5-27] Generally data were collected for each control group and treated mice comprising a batch of 5 to 6 mice for each fixation timepoint (several fixation intervals and three sets of replicates were considered in most experiments). The control sample(s) and homeopathic medicine were given by oral gavage.

Cytogenetical

Mice have 20 pairs of acrocentric chromosomes in their somatic cells, which at metaphase provide ample opportunity to study structural and numerical aberrations inflicted by mutagens, toxicants, and carcinogens (Figure 1). The ability of any agent to stimulate repair/protection of chromosomes from aberrations is an acceptable parameter of study for clastogenic or genotoxic effects. Micronuclei induction bears testimony to the fact that chromosomes or parts thereof have been broken, and in a last bid to repair/protect the cell from death, micronuclei containing the broken part are pinched off. The induction of micronuclei is also a potent parameter of study for genotoxic effects of chemical or physical mutagens. Similarly, abnormality in sperm head morphology induced by toxins and mutagens is a good indicator of spermatotoxic effect. Similarly the mitotic index refers to the change in mitotic activity, which may be slowed (in case of CNS damage by irradiation) or increased (mostly in case of induction of cancer by carcinogens).

Single-cell gel electrophoresis (Comet assay): The damage inflicted by a mutagen/carcinogen/toxin on the DNA of a cell can be assessed by the Comet assay, ascertaining the length of the 'Comet tail' in the affected nucleus. A longer tail implies greater fragmentation of the nucleus exuding DNA as the longer tail (Figure 1).

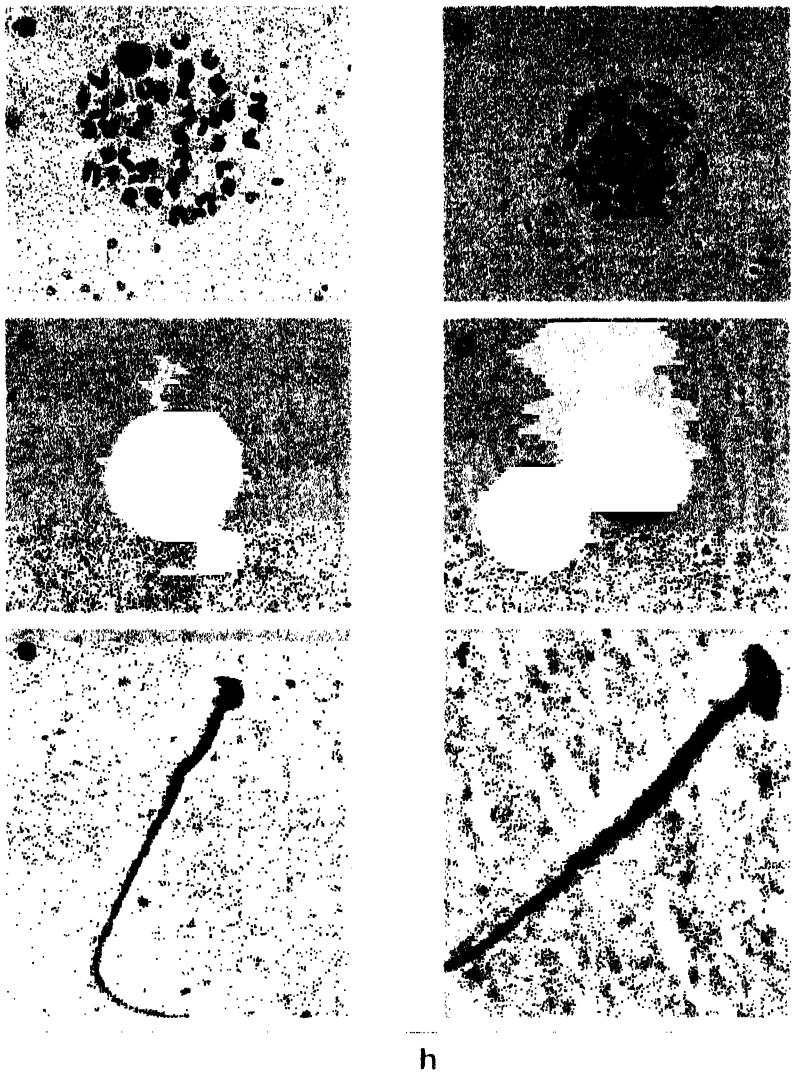

Figure 1 Photomicrographic representation of chromosome spreads (a = normal, b = aberrated), micronuclei (c), dividing cell (d) sperm head (e = normal, f = abnormal) comet tail (g = normal, h = extended tail).

Fluroscence activated cell sorting (FACS): Mitotically dividing cells, like the bone marrow cells, skin cells or liver cells were subjected to FACS for the determination of the exact stage of the cell cycle where the mutagen/carcinogen acts. Thus, carcinogens tend to enhance the cell cycle by stimulating the division mechanism of the cell through various mechanisms and FACS data (Table 1) provides evidence of an ability to arrest this enhanced cell divisional activity. Similarly, if a drug shows anti-cancer activity, it is expected to arrest the division at the pre- or post-synthetic phase. FACS data analysis can provide evidence of the stage of cell cycle targeted.

DNA gel electrophoresis for ladder and RT-PCR for mRNA expression: We used standard gel electrophoretic methods for analysis of DNA ladder[28] and a standard method for analysis of cDNA from RT-PCR of mRNA[29] to measure the expression level of specific gene(s), for instance PCNA, p53 (Figure 2) and the 'housekeeping' gene, β-actene.

Biochemical toxicity biomarkers: Certain biochemical parameters are important in ascertaining toxicity or functional status in certain important organs like liver, spleen, kidney, or heart. The activity of enzymes such as acid phosphatase (AcP), alkaline phosphatase (AlkP), AST and

Table 1 (a) Showing data of Caspase-3 activity, cytogenetical damage. and cell cycle analysis of DMBA treated and other groups of mice (skin cancer) (unpublished data)

Series	*Caspase-3 activity (μM/mg protein*	*Cytogenetic damage*							*Cell cycle analysis by FACS*			
		% of major CA	*% of other CA*	*% of total CA*	*% of MN*	*% of MI*	*% of SHA*	*% of DNA break (Comet assay)*	*SubG1 (%)*	*G1 (%)*	*S (%)*	*G2/M (%)*
Normal	25.78 ± 0.074	2.45	2.15	4.60 ± 0.40	0.225 ± 0.08	2.12 ± 0.04	1.26 ± 0.02	13.1 ± 3.72	32.07 ± 0.375	32.73 ± 0.565	17.44 ± 1.24	2.16 ± 0.020
DMBA + Croton oil	14.36 ± 0.061	12.1	9.7	21.8 ± 0.54	1.13 ± 0.09	7.39 ± 0.04	2.60 ± 0.02	72.73 ± 4.33	23.39 ± 0.005	23.56 ± 0.560	25.73 ± 0.610	6.62 ± 0.375
DMBA + Croton oil + Alcohol	12.8 ± 0.058	14.0	10.3	24.3 ± 0.67	1.22 ± 0.05	6.9 ± 0.03	2.80 ± 0.03	95.63 ± 5.45	22.15 ± 0.105	28.41 ± 0.410	28.74 ± 0.640	4.76 ± 0.040
DMBA + Croton oil + Kali ars-30	16.71 ± 0.769†	8.7	6.3	15.0 ± 0.330‡	0.68 ± 0.0‡	3.8 ± 0.05‡	1.76 ± 0.05‡	53.93 ± 2.01†	34.61 ± 0.395‡	30.43 ± 0.42*	22.44 ± 0.43†	2.26 ± 0.415†

(b) Showing data of cytogenetical damage by Acrylamide (unpublished data)

Series	*Cytogenetical damage*				
	% of total CA	*% of MN*	*% of MI*	*% of SHA*	*% of DNA break (Comet assay)*
Normal	4.67 ± 0.15	0.22 ± 0.03	0.76 ± 0.05	0.60 ± 0.05	12.45 ± 0.10
Acrylamide	16.67 ± 0.23	0.75 ± 0.03	4.26 ± 0.06	2.36 ± 0.05	75.25 ± 0.15
Acrylamide + Alcohol	17.67 ± 0.31	0.83 ± 0.03	5.0 ± 0.05	2.46 ± 0.06	63.56 ± 0.11
Acrylamide + Myrica	10.67 ± 0.40‡	0.56 ± 0.03‡	2.5 ± 0.06‡	1.16 ± 0.04‡	35.24 ± 0.20‡

Values are expressed as mean ± SE. Comparisons are made between carcinogen treated and homeopathic treated mice.
* $p < 0.05$.
† $p < 0.01$.
‡ $p < 0.001$.

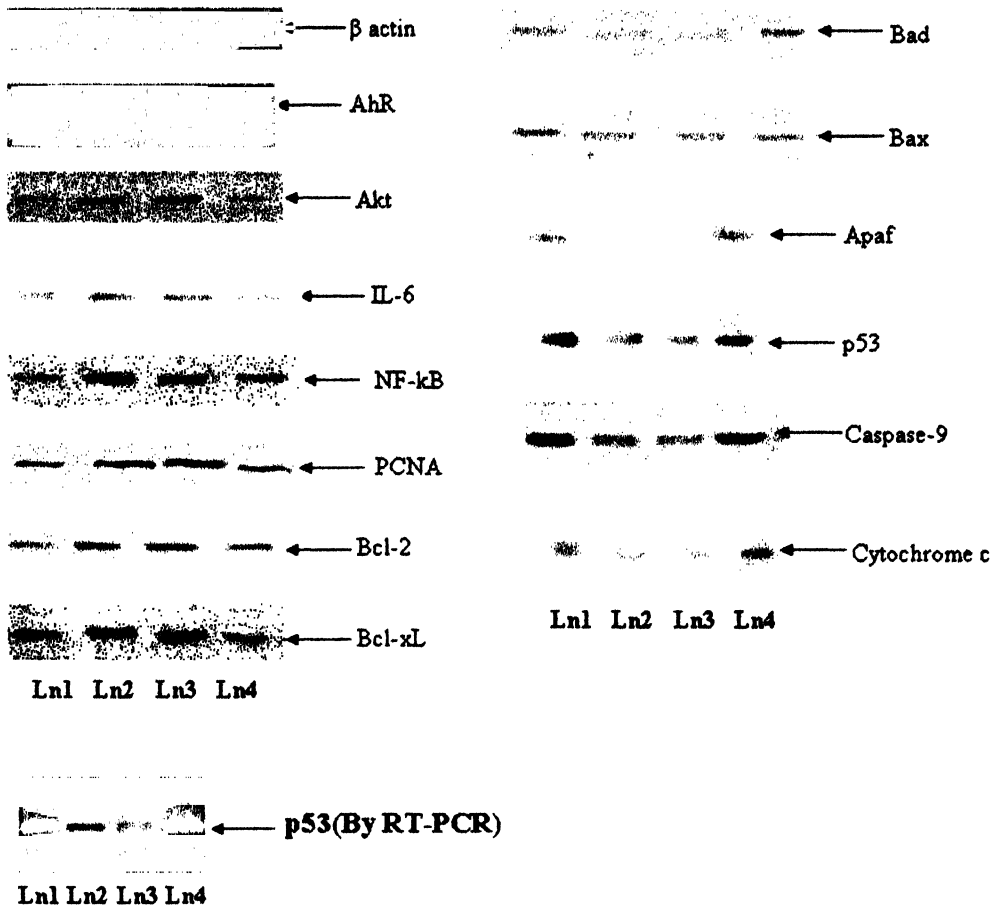

Figure 2 Immunoblots of β-actin, Ahr, Akt, IL-6, NF-kB, PCNA, Bcl-2, Bcl-xL, Bad, Bax, Apaf, and p53, cytochrome c, Caspase-9 and also the RT-PCR analysis of p53 protein. Ln1 – normal; Ln2 – DMBA; Ln3 – DMBA + croton oil + alcohol; Ln4-DMBA + croton oil + Kali Ars 30.

ALT are linked to hepato-cellular injury or necrosis in striated muscle tissue.[30-34] Determination of ALT activity has been considered a relatively sensitive indicator of hepatic damage, and release of ALT from the cytosol may be secondary to cellular necrosis or a result of cellular injury with membrane damage and bleb formation.[35] The increase in the activities of AcP, AlkP, AST and ALT is often associated with the overall increase of the toxicity level and *vice versa*, and can implicate the state of toxicity associated with tumor growth.[32] Gamma glutamyl transeferase (GGT) also provides a sensitive indicator of hepato-biliary dysfunction. A fairly strong correlation exists between the increase of lipid peroxidation (LPO) and decrease of reduced glutathione (GSH) as a result of toxicity. In LPO, a cascade of peroxidative reactions leads to the destruction of lipid liberating malonaldehyde, which ultimately affects membrane structure.[17,18] If hepatotoxicity occurs, free radicals are generated. GSH and Superoxide Dismutase (SOD) have anti-oxidant activity (Table 2) which is accomplished by scavenging free radicals. GSH is also known to have an important role in regulation of cellular proliferation and cellular defense. Most of the interest of glucose-6-phosphate dehydrogenase (G-6-PD) focuses on its role in the erythrocytes. Thus G-6-PD is correlated to function of glutathione and GSH contents can provide additional information on the state of oxidative stress and its modulation may indicate its cellular protective activity. There are some mitochondrial and peroxisomal enzymes that can be used as toxicity biomarkers, including catalase, succinate dehydrogenase and glutathione reductase (GR.).[36,37] The sub-cellular environment can be depicted by the activities of these enzymes.

Histological and electron microscopic studies: Standard histological and electron microscopic staining methods were used.[17,19] Tissue damage and sub-cellular changes due to carcinogens or mutagens can provide important information on whether an agent can protect or help in the repair and recovery process.

Gel electrophoretic protein profile: Every tissue exhibits a typical gel electrophoretic protein profile that often is modified by toxic effects on DNA and protein structure. Therefore, the change in protein profile due to toxic chemical/mutagen treatment and its protection/repair after drug treatment can focus on the ability of the drug to ameliorate damaging effect of the mutagen/toxicant on the profile.

Signal proteins and gene expressions: Signal proteins are useful indicators of the pathway of signal transduction and mechanism of action of both the toxicants/carcinogens as well as of treatments. Most of these signal proteins also depict up- or down-regulation, directly demonstrating the extent of expression(s) of specific gene(s) (Figure 2). This often is considered significant finding in support of

Table 2 Showing data of enzyme assay (unpublished data)

Series	Enzyme assay				
	SOD	CAT	LPO	GST	GSH
Normal	17.31 ± 0.271	36.05 ± 0.240	27.47 ± 0.140	2.58 ± 0.255	35.97 ± 0.260
DMBA + Croton oil	9.77 ± 0.060	26.12 ± 0.240	42.03 ± 0.325	1.78 ± 0.020	23.25 ± 0.125
DMBA + Croton oil + Alcohol	8.30 ± 0.085	23.71 ± 0.020	44.98 ± 0.225	1.6 ± 0.020	22.25 ± 0.060
DMBA + Croton oil + Kali Ars-30	14.03 ± 0.135*	29.07 ± 0.040*	40.02 ± 0.240*	1.98 ± 0.035*	27.88 ± 0.119*

Values are expressed as mean ± SE. Comparisons are between untreated, carcinogen administered no treatment, carcinogen administered succussed alcohol treated, and carcinogen administered homeopathic-treated mice.
* = $p < 0.001$.

expression of specific proteins detected by monoclonal antibodies, pointing to the activity of specific gene(s).

Immunohistochemical and immunofluorescence localizations: Localization of particular signal proteins in cell/tissue/receptor is important in determining activity of proteins with the help of specific antibodies tagged with fluorescing dyes (Figure 3).

Physico-chemical properties: 13C and ^{1}H NMR, Mass Spectrometry, UV-spectrophotometry, HPLC, COSY and FTIR analyses of some plant extracts used as homeopathic remedy were conducted to chemically isolate and characterize the active principle(s) showing biological action like anti-cancer property through their ability to induce apoptosis in cell free system.

Statistical analysis: All data were subjected to appropriate statistical analyses, mostly by Student *t*-test or one way ANOVA for determination of significance levels, if any, between treated and control.

Results

Significant results obtained in our laboratory have been presented in brief (Tables 1–3) while the results in greater detail may be had from the original published papers cited in Table 3.

Radiation-induced damage and protection/repair

Mice were exposed to whole-body X-irradiation at different sub-lethal doses and the efficacy of several potentized homeopathic medicine in ameliorating chromosome aberrations, micronuclei induction, and mitotic index of bone marrow cells and sperm head anomaly were studied at several time points.[3,5,8,13–16,38] Different homeopathic medicines, known to have ameliorative action against shock and injury, like *Arnica montana*, 30C, 200C, Ruta graveolens 30C, 200C, Ginseng 30C, 200C, etc. were found to ameliorate X-ray induced genotoxicity in mice, as compared to controls. Subsequently, more detailed study was carried out on X-ray induced genotoxicity in mice and its amelioration by some homeopathic medicine[4,22,23,39] including some other potentized homeopathic medicine like Hypericum 200C, *X-ray* 30C, etc. Pre- and post-treatment with the homeopathic medicine generally showed better efficacy than only post-treated or only pre-treated mice, in that order. Since repair mechanism of X-ray induced damage has been more thoroughly studied in various organisms including mice earlier, and the role of several specific repair genes involved is already known, this study assumes great importance because the homeopathic dilutions that produced excellent protective effects were diluted much above Avogadro's limit. *Arnica montana* 30C also protected cytogenetic ill-effects of over-dosed ultra-sound in mice.[40]

Chemical-induced damage and homeopathic protection/repair

The widespread occurrence of arsenic poisoning through ground water contamination in West Bengal, India, and Bangladesh led us to explore whether homeopathic treatment could ameliorate the effects of arsenic poisoning. For this, we made a series of laboratory experiments using multiple protocols[9–14,21,24,41] in mice intoxicated with single injection of a sub-lethal dose of arsenic trioxide and also followed by repeated injections of Arsenic trioxide[24–26] and treating them with a potentized *Arsenicum album*-30C, derived from homeopathically diluted Arsenic trioxide, based on the homeopathic principle 'like cures like'. Homeopathic *Arsenicum album*-30C and 200C positively modulated multiple toxicity parameters and provided evidence for its ability to ameliorate arsenic induced toxicities in mice. Subsequently, the efficacy of this homeopathic remedy was tested on human in field trials[42–44] and was found to have considerable ameliorative effects.[45] Incidentally, toxicological effects have been found to be reduced in experimental mice by using homeopathic potentized remedies like *Cadmium sulphuricum* against Cadmium chloride.[46,47] *Mercurius solubilis* against Mercuric chloride *Stannum* 30C against Stannous chloride,[48] etc. as compared to suitable controls.

Carcinogenesis: liver

Induction of liver cancer through chronic feeding of the azo dye, p-dimethylaminoazobenzene (p-DAB) and Phenobarbital has been used as a tool to investigate the chonology of events leading to cancer as well as to examine the effects of possible anti-cancer treatments in mice. Several homeopathic medicines have been tested in our laboratory using a series of scientifically validted biochemical protocols (toxicity biomarkers) supported with histological and

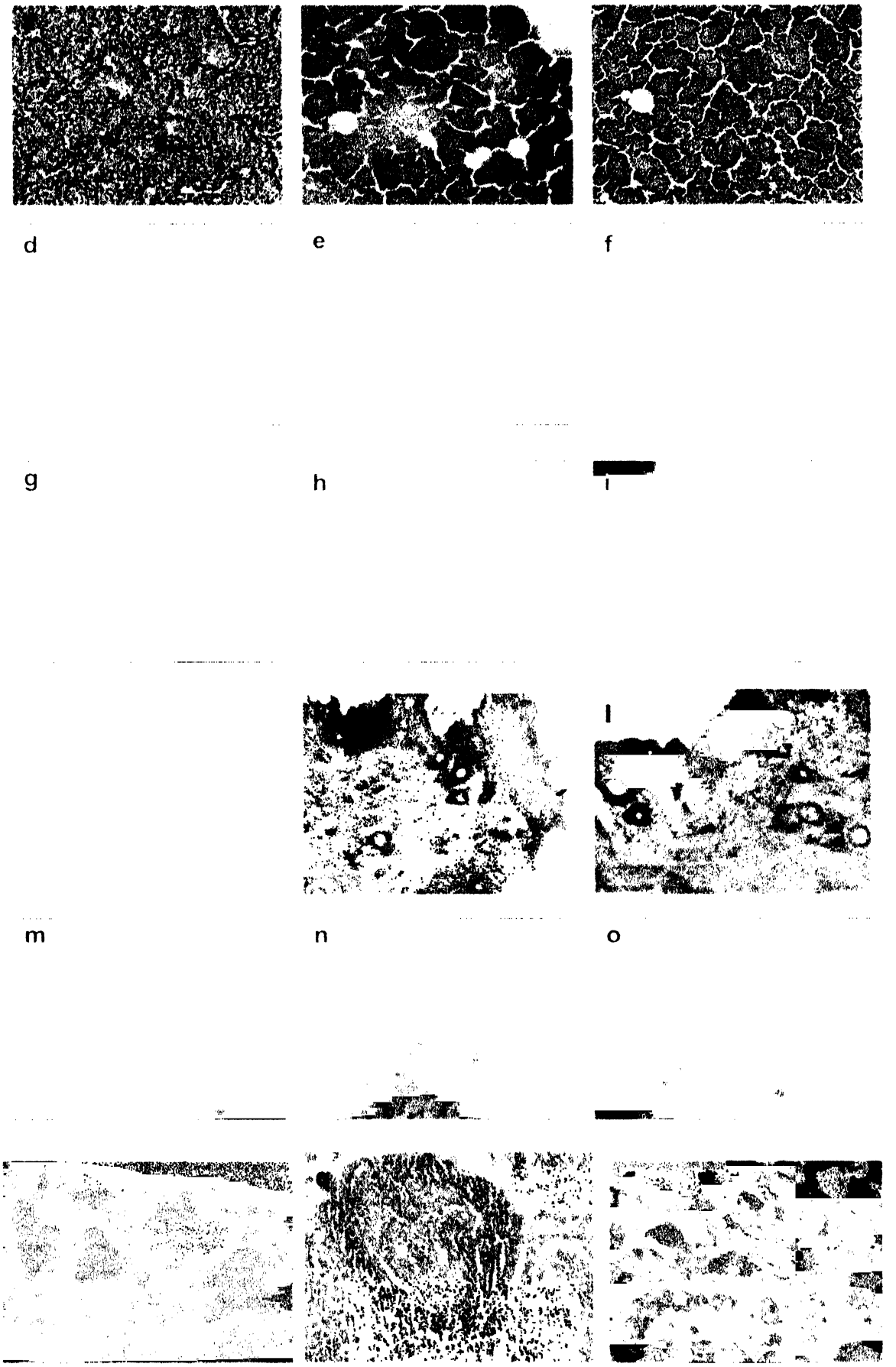

Figure 3 (a–c) Histology of liver stained with Haematoxylin/Eosin (a = normal, b = Acrylamide + alcohol, c = Acrylamide + Myrica treated); (d–i) immunofluorescence expression of Bcl-2 protein (d = normal, e = Acrylamide + alcohol, f = Acrylamide + Myrica treated) and Bax protein (g = normal, h = Acrylamide + alcohol, i = Acrylamide + Myrica treated); (j–l) Histology of skin stained with Haematoxylin/Eosin (j = normal, k = DMBA + croton oil + alcohol, l = DMBA + croton oil + Kali Ars 30 treated) and (m–o) immunofluorescence expression of PCNA protein (m = normal; n = DMBA + croton oil + alcohol; o = DMBA + croton oil + Kali Ars 30 treated); (p–r)Histology of lung stained with Haematoxylin/Eosin (p = normal, q = B(a)P + Alcohol, r = B(a)P + Silicea 6).

Table 3 A summary of major works on homeopathy carried out in mice in laboratory of A.R. Khuda-Bukhsh

Citation	*Descripion*	*Findings*
Khuda-Bukhsh *et al.*[50]	The efficacy of Secale Cor 30C tested on mice administered DMBA/Croton oil to induce skin papilloma by analyzing results of cytogenetical endpoints, FACS, and expression of signal proteins like AhR, p53, PCNA, Akt, Bcl-2, Bcl-xL, Bad, Bax, NF-kB, Apaf, IL-6, Cytohrome c, Caspase-3 and Caspase-9 and *immunohistochemical and immunofluorescence* localization.	*Secale cor* 30C showed positive results in amelioration of skin papilloma
Bhattacharyya *et al.*[27]	A synthetic scopoletin (active principle of Gelsemium sempervirens mother tincture) analogue, 4-Methyl-7-hydroxy coumarin) tested on mice administered DMBA/ croton oil to induce skin papilloma and the effects of treatments tested by analyzing cytogenetical endpoints, Comet assay, FACS, and expression of signal proteins like AhR, p53, PCNA, Akt, Bcl-2, Bcl-xL, Bad, Bax, NF-kB, Apaf, IL-6, Cytohrome c, Caspase-3 and Caspase-9, and immunohistochemical and immunofluorescence localization.	Scopoletin showed positive results.
Banerjee *et al.*[25]	To test efficacy of Arsenicum Album 200C in amelioration of mice receiving repeated injections of Arsenic trioxide. Test protocols included assay results of AST and ALT, GR, catalase, succinate dehydrogenase, SOD, and reduced glutathione contents. Electron microscopies (both SEM and TEM) and gelatin zymographies of matrix mettaloproteinases of liver tissues also conducted.	Results showed positive ameliorating ability of *Arsenicum album* 6C, 30C and 200C.
Pathak *et al.*[76]	The efficacy of ethanolic spore extract of *Lycopodium clavatum* (homeopathic mother tincture) was tested in mice chronically fed p-DAB to induce liver cancer. P-DAB increased activities of AcP, AlkP, levels of blood glucose and cortisol, and decreased the activities of GR, succinate dehydrogenase, blood cholesterol, hemoglobin contents and levels of serum estradiol and testosterone in mice,	*Lycopodium* was associated with positive modultations.
Biswas *et al.*[18]	Ethanolic whole plant extract of *Chelidonium majus* tested for its possible anti-tumor, hepato-protective, and anti-genotoxic effects in p-dimethylaminoazobenzene (p-DAB) induced hepatocarcinogenesis in mice. Several cytogenetical endpoints, biochemical toxicity biomarkers and histology of liver through ordinary, scanning and transmission microscopies were considered.	Results indicate positive anti-tumor, anti-genotoxic and hepatoprotecive effects of *Chelidonium majus*.
Pathak *et al.*[7]	To examine if *Lycopodium*-200 has demonstrable anti-cancer activities in mice induced by carcinogens p-DAB and PB.	Positive results obtained in respect of anti-tumor, anticlastogenic, hepato-protective, anti-spermatotoxic endpoints.
Banerjee *et al.*[26]	To examine if *Arsenicum album* 200C could protect mice receiving repeated doses of arsenic trioxide from genotoxic effects.	*Ars alb* 200C positively modulated the cytogenetical endpoints indicating its protective potential.
Banerjee *et al.*[24]	To evaluate the comparative efficacy of two potentized homeopathic remedies of *Arsenicum album* (*Ars. alb*-6C and 30C) in combating chronic arsenic toxicity induced by repeated sub-lethal injections of Arsenic trioxide in mice.	Both potencies showed amelioration of arsenic toxicity, *Ars alb* 30C had slightly better efficacy.
Bhattacharjee *et al.*[19]	To examine if potentized homeopathic *Natrum sulph* 200C has protective potentials against p-DAB induced hepatocarcinogenesis in mice.	*Natrum sulph* 200C showed protective potentials in reducing incidence of tumor in liver and showed positive modulations of other parameters like cytogenetical, biochemical, histological.
Pathak *et al.* 2006.[5]	To test protective potentials of *Lycopodium* 30C in mice chronically fed carcinogens by deploying some cytogenetical and biochemical parameters.	*Lycopodium* 30C showed both anti-genotoxic and anti-cytotoxic effects.
Biswas *et al.*[17]	To test efficacy of *Carcinosin*-200 treated alone and in combination of *Chelidonium*-200C in combating induced hepatocarcinogenesis in mice.	*Chelidonium*-200 when administered alone showed considerable ameliorating effect, but the conjoint administration of *Chel*-200C and *Carcinosin*-200C appeared to have slightly greater protecting effect.
Biswas *et al.*[16]	To evaluate protective effects of *Chelidonium*-200C against azo dye induced genotoxicity in mice.	The frequencies of CA, MN, SHA, MI was less as compared to azo dye fed mice and biochemical parameters supported the findings as well.
Datta *et al.*[47]	Effect of pre-feeding, post-feeding and combined pre- and post-feeding of homeopathic drugs *Mercurius solubilis* against Mercuric chloride induced genotoxic effects in mice.	*Merc sol* 200C showed better protective effect in the combined pre- and post- fed mice.
Biswas *et al.*[15]	To evaluate comparative effects of *Chelidonium* 30C and 200C against hepatocarcinogenesis in mice.	Both potencies of *Chelidonium* exhibited anti-tumor, anti-genotoxic and anti-cytotoxic effects, *Chel*-200C having a little better efficacy at longer fixation intervals.

Table 3 (*Continued*)

Citation	*Descripion*	*Findings*
Chakraborty *et al.*[40]	To evaluate protective role of *Arnica* 30C and Actinomycin D against shock and injury inflicted by ultra sonication in mice.	*Arnica* 30C showed appreciably reduced genotoxicity as against Alcohol 30 and distilled water treated controls.
Kundu *et al.*[11]	To test efficacy of *Arsenicum album* 30C in alteration of pathological changes, protein profiles, DNA and RNA content produced by arsenic trioxide in mice.	*Ars.* a 30 has potentials in reducing arsenic induced damage to protein and nucleic acids and the mechanism of action of homeopathic medicine therapy expression of regulatory genes inferred.
Kundu *et al.*[12]	To evaluate protective effects of *Arsenicum album*-30C in reducing cytotoxic effects produced by arsenic trioxide in mice.	The mice treated with homeopathic medicine showed positive results of tissue recovery both in terms of enzymatic and histological changes.
Mitra *et al.*[10]	To examine if *Arsenicum album*-30C can reduce arsenic toxicity in mice in terms of alterations of body weight, tissue weight and total protein.	Arsenic treated mice receiving the homeopathic treatment significantly increased tissue weight, protein content as well as mean body weight.
Mitra *et al.*[9]	To test efficacy of *Arsenicum album*-30C in reducing toxic effects produced by intramuscular injection of As_2O_3 in mice – on rate of accumulation of arsenic in certain vital organs.	*Ars alb* 30C effectively anti-doted arsenic poisoning in arsenic treated mice and significantly reduced accumulation of As in different tissues studied.
Banik and Khuda-Bukhsh[23]	To examine if micro doses of Ginseng 30C can positively modulate cytogenetical and hematological parameters in mice exposed to whole-body X-irradiation.	Positive amelioration by administration of *Ginseng* was noted.
Khuda-Bukhsh and Banik[8]	To study comparative efficacy, if any, of Ginseng, 6C, 30C and 200C in modulation of cytogenetical parameters like chromosome aberrations, micronuclei and mitotic indices of mice exposed to whole-body X-irradiation of 100 and 200 rads, respectively.	All three potentized Ginseng reduced cytogenetical damage, but no clear-cut difference could be discernible regarding differential efficacy of different potencies.
Khuda-Bukhsh and Maity[5]	To examine if *Ruta graveolens* 30C and 200C can modulate cytogenetical effects produced by X-irradiation.	Both produced ameliorative changes.
Khuda-Bukhsh[3]	If *Arnica montana* 30 and 200C, Hypericum 30, X-ray 30C and Ruta 30C could modulate effects of whole-body X-irradiation.	All potentized homeopathic medicines ameliorated X-ray induced cytogenetic damages.
Khuda-Bukhsh *et al.*[38]	To examine if *Arnica montana* 30 can modulate X-ray induced chromosome aberrations in mice.	Positive modulation.

electron microscopic findings in the mice model induced to develop liver cancer.[6,7,15,16,19,49] The majority of well selected homeopathic medicines tested in our laboratory yielded positive results in preventing development of liver tumors in mice chronically fed liver carcinogens, compared to controls: 20–60% of mice responded positively showing minimal growth of tumor, if at all any. Most of these homeopathic medicines (claimed in homeopathic literature to have profound curative action on liver) tested, including *Chelidonium*, *Lycopodium*, *Carduus*, *Hydrastis*, etc. also showed varying degrees of anti-oxidative stress effects. Recently, the effects of chronic feeding of another toxic chemical, Acrylamide, have been investigated in mice and the efficacy of the mother tincture of *Myrica* has been tested to be positive with multiple protocols (Table 1(b)).

Carcinogenesis: skin

Recently, we conducted studies to determine the effects, if any, of homeopathic medicines, including *Secale cornutum*, *Gelsemium sempervirens*, *Kalium arsenicosum*, *Arsenicum album*, etc. and some of their active principles in amelioration of DMBA/croton oil induced skin papilloma. Varying degrees of protective effects were observed with all the homeopathic remedies mentioned above except for *Ars alb* which did not show a significant positive result. This work is supported by biochemical, cytogenetic and cell signaling parameters. Several signal proteins have been found to be altered by treatment a novel finding.[50] Techniques icluding immunofluorescence, Western blots etc have also support the work (Figures 2 and 3).

Carcinogenesis: lung

Experiments are presently underway to examine the efficacy of several homeopathic medicines including *Senega*, *Silicea*, and *Phosphorus* in Benz(a)pyrene induced lung cancer in mice. Of these, mother tincture of *Senega* showed the best protective effect, *Silicea* 6C was relatively less effective, while *Phosphorus* had effects no different from control (results unpublished) (Figure 3; Table 2).

Immunomodulation

Studies have recently been conducted on *in vivo* response of mice to chronic arsenic intoxication in respect of peritoneal macrophage ROS generation, modulation of several toxicity biomarkers, mitochondrial oxidative stress enzyme, liver cell cycle, mitochondrial membrane potential (*in vitro* with RAW 264 cells) etc. Results are encouraging: pre-treatment with *Arsenicum album*-30C positively modulates these markers, suggesting it is effective as a preventive agent against arsenic intoxication. Further immuno-modulatory studies are now in progress.

Discussion

Our initial results with X-ray induced chromosomal aberrations and their protection/repair by several potentized homeopathic medicines generally used against shock and

injury, compared to succussed alcohol treated controls, led us to look for the possible mechanism(s) by which chromosome/DNA damage is repaired after radiation injury with the available or intrinsic mechanisms known to be present in mice. As control mice failed to show any detectable protection/repair and the homeopathic remedies consistently showed positive results, we wondered how these ultra-high diluted remedies could bring about such changes manifested in the repair of chromosomes and sperm head morphology. Literature[1,2] suggest that certain specific genes are actively involved in the repair process. Subsequently, certain potentized homeopathic medicines were also found to repair/protect chromosomal and nuclear damage (also DNA damage) induced by certain chemical mutagens and toxins. This is not possible without the involvement and acceleration of functioning of the repair 'genes'. Again, in contrast to homeopathic remedies, the succussed alcohol failed to show up any protection/repair ability.

Various other patho-physiological studies involving many biomarkers (mostly proteins transcribed from DNA and translated), known to have positive effects on the recovery process, were also found to be positively modulated by the homeopathic remedies while control failed to show any such positive outcome. It appears that homeopathic remedies can positively modulate features associated with the development of cancer (an outcome of failure in gene regulatory mechanism owing to mutations causing transformation of proto-oncogenes to oncogenes) and prevent various types of tumors/cancers in mice subjected to chronic treatment with carcinogens. Corresponding ultra-structural changes were demonstrated in vital organs like liver for the action of the remedies claimed to have profound protective action on liver. Favorable changes of many other parameters were induced by potentized homeopathic remedies while alcohol controls consistently failed to produce any favorable changes in mice.

Thus, the homeopathic remedies were found to be effective on various parameters of study (under regulatory control of certain known genes) and on different organisms.[51] As all the parameters of study tested are under genetic control, it would appear that corrective activities of different regulatory genes initiate the recovery process. This would imply that potentized homeopathic medicines though devoid of any drug molecule, were still capable of acting in a multidirectional manner, which led to the idea that they could trigger the activity through their regulatory action on master gene(s), that in a follow up cascade of reactions activated/deactivated downstream genes responsible for the recovery process.

Results obtained by us and others suggest that well selected homeopathic remedies accelerate cellular and subcellular activities that are expected to be associated with a recovery process. Notwithstanding the fact that some animals did not respond as favorably to treatment as others (perhaps due to different genetic predispositions?), these results indicate the ability of the drug to address the problem of a specific metabolic disorder causing the disease symptoms, while succussed alcohol control apparently did not have the ability. But how this was precisely accomplished by the ultra diluted homeopathic remedies remains a scientific enigma.

At the present state of our knowledge, the mechanism of action of homeopathic medicines diluted beyond Avogadro's limit is extremely difficult to comprehend without the help of some working hypotheses. We will by and large restrict ourselves to the area of its biological action, mentioning briefly some other relevant physico-chemical aspects. Firstly, to understand the mechanism of action of the potentized homeopathic medicines, one has to satisfactorily answer the problem of transfer of medicinal property of the original drug substance to and retention of the same by the vehicle. Several working hypotheses have been proposed.[52-64] The leading current proposals for the mode of action of such 'ultra molecular' dilutions is that water is capable of storing information relating to substances with which it has been in contact and subsequently can transmit this information to pre-sensitized biological system. The process is believed to be mediated by structural modification of water, analogous to storage of information by magnetic media.[54] Such 'information' is retained in physical, rather than chemical form. Thus molecular architecture of water has a key role to play in understanding homeopathic mechanism of action and has been studied.[57,65,66]

Comprehensive reviews are also available that deal with *in vivo* and *in vitro* experiments with animal models in relation to various immunological, cardiovascular and molecular aspects and various mechanisms suggested to explain the results[51,54,67-70] to understand how the ultra-high dilutions of homeopathic remedies may exert their action. Khuda-Bukhsh[71-73] proposed a hypothesis based on various evidences that potentized homeopathic medicines act through regulation of gene expression. The possible pathways and sites of action have also been discussed by this group[21,72,74]. According to this hypothesis, homeopathic remedies carry specific 'signals' that can be identified by specific receptors and can act as a trigger to turn 'on' or 'off' some relevant genes, initiating a cascade of gene actions to alter and correct the gene expressions that went wrong to produce the disorder/disease. At the present state of our knowledge emanating from the various toxicological experiments, particularly from the induced cancer experiments, it appears that one way that homeopathic medicines could act is mediated through cytokine signaling, particularly in view of the fact that certain potentized homeopathic medicines modulate signal proteins like NF-kB and IL-4,6.[50] Thus, administration of a potentized homeopathic medicines can elicit response in signal proteins and can either up-regulate or down-regulate such signal proteins. A global microarray after a homeopathic medicines administration in a disease state quite provided evidence for modulated expression of a large number of genes.[75]

In higher eukaryotes like mammals, the regulation of gene expression is a very complex phenomenon. In principle, the expression of genes might be regulated at any one of several stages. Therefore, more work in this area is necessary to precisely understand the actual molecular mechanism *via* various steps from receptor binding/activation through gene regulation and expression at the target tissue/organ. During this process, the roles of 'activators', 'enhancers', 'gene silencing', 'phosphorylation/dephosphorylation, etc have

to be properly assessed to understand the actual molecular mechanisms involved in transmission of 'information' of the homeopathic remedy down to the 'execution' level for active recovery process: Activation of structure – initiation of transcription – processing the transcript – transport to cytoplasm – translation of mRNA. Each signal is communicated to the gene by a separate activator (signal recognition particle).[2] Signals are often communicated to transcriptional regulators through signal transduction pathways.[1] However, how a homeopathic medicines can elicit response in a cell receptor and bind with the receptor is not known. The problem of ligand-binding to receptors, if ultra diluted homeopathic remedies actually have to bind on receptors to elicit specific response has to be resolved. One possibility is that homeopathic medicines, during the 'dynamization/potentization' process, interact with nanoparticles that eventually may have some role in tagging onto some proteins, that eventually act as ligand[68,27]; this hypothesis needs to be further explored. Likewise, there are signal transducers and activators of transcription (STAT) and mitogene activated protein (MAP) kinase pathways activated by ligand (a cytokine) in which phosphorylation and dephosphorylation of kinases occur producing activity in genes downstream as a cascade of reactions.

Gene expression can be controlled by 'signals' received by a cell from its environment. For example, the presence of lactose activates the transcription of the lac operon in *E. coli*, while viral infection activates the expression of β-interferon gene in mammals.[1] Interestingly, in our recent experiment on *E. coli* subjected to low dose of arsenic, *Glucose* 30C and *Arsenicum album*-30C showed modulatory effect in their glucose uptake (results unpublished). Generally speaking, the strategies that are used to instruct genetically-identical cells to express distinct sets of genes and thereby perform different sets of function include mRNA localization, cell-to-cell contact and signaling through the diffusion of a secreted signaling molecule. Microarray assays can therefore help further in the analysis of gene expression profiles of experimental organisms administered micro doses of homeopathic medicines.

The evidences that support gene regulatory hypothesis are:

i) Modern techniques have convincingly demonstrated the action of several potentized homeopathic medicines diluted beyond Avogadro's limit in modulating significantly many dependable parameters compared to suitable controls. Further multiple parameters change in the same time frame, which without a cascade of gene actions is difficult to explain.
ii) The homeopathic remedies are diluted to such an extent that there cannot be physical existence of a single molecule of the original substance that can chemically react. But there are demonstrable modulations in almost every endpoint which can be attributed to the action of a homeopathic medicines diluted beyond Avogadro's limit.
iii) In contrast to the presence of molecules in orthodox drugs and which are absent in the potentized homeopathic remedies, the possibility that the homeopathic drug may indeed carry 'molecular imprints' or definite 'signal/information' of the original molecule[77] that can be deciphered by the cells' receptors, cannot be ruled out. Further, the ability of potentized homeopathic medicines to modulate expression of signal proteins and receptor proteins (like AhR, PCNA) may provide additional evidence for their ability to influence on the regulatory genes. However more accurate and specific studies should be carried out to map exactly the first point of cellular changes triggered by homeopathic treatment.
iv) Nanoparticles have been shown to have effects on the physico-chemical property of the homeopathic dilutions,[27] that may indicate some role of the nanoparticles during the potentization process of homeopathic medicines.
v) All parameters studied to date are gene controlled.
vi) In presence of Actinomycin D, a transcription blocker, a potentized homeopathic medicines failed to act.[14,40]
vii) Homeopathic dilutions have demonstrable action on plants which lack any central nervous system.[51]

Thus, homeopathy research in mice yielded many suggestive information like: i) the medicinal action can be demonstrated compared to controls; ii) much of the mechanism of action can now be understood on the basis of scientific evidences, iii) these researches are opening up new avenues of research in other systems and models to pave the way for a better understanding of the mechanisms and pathways of homeopathic medicine action.

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