

## ORIGINAL PAPER

# Impact of homeopathic remedies on the expression of lineage differentiation genes: an *in vitro* approach using embryonic stem cells



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**Background:** Well-documented studies of the potential effects and safety of homeopathic medicines in pregnancy are required. In this study, specific genes were studied which could serve as biomarkers for specification of three lineages to predict the safety of homeopathic remedies using mouse embryonic stem (ES) cells. Thus, the present work was to study the effects of homeopathic remedies taken during pregnancy using ES cells as the model.

**Methods:** Mouse ES cells were exposed to 30C potency of *Nux Vomica* and *Sepia*, which are homeopathic medicines prescribed for the management of pregnancy related symptoms. Cytotoxicity studies were done using a modified Embryonic Stem cell test (EST). The expression levels of key genes and proteins were analyzed using real time polymerase chain reaction and immunocytochemistry, respectively.

**Results:** Homeopathic treatment led to modulations in the expression of certain lineage specific genes but this difference was not significant with respect to solvent control and showed normal differentiation as demonstrated by the expression of  $\alpha/\beta$  MHC and  $\alpha$ -actinin proteins in the differentiated ES cells.

**Conclusions:** Our study for the first time has shown the feasibility of using ES cells in the developmental toxicity testing of remedies. The results suggest that they are not associated with developmental toxicity. *Homeopathy* (2016) 105, 148–159.

**Keywords:** Homeopathy; Embryonic stem cells; Differentiation; Pregnancy; Developmental toxicity

**Abbreviations:** ES = embryonic stem; EB = embryoid body; Nes = nestin; ND200 = neurofilament 200 kDa; Afp = alpha fetoprotein; GAPDH = glyceraldehyde-3-phosphate dehydrogenase

## Introduction

Homeopathic medicines are considered to be safe because of the belief that at dilutions which are conventionally used for treatment were associated with less adverse effects.<sup>1</sup> Homeopathic medicines are prepared by the process of dynamization, in which a miniscule quantity of the original natural medicinal essence is necessary when manufacturing these medicines. These remedies are believed to be safe and were given even to drug sensitive groups like pregnant

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women, babies and elders. Some conventional medicines such as zantac, emetrol, pepcid, phenergan are prescribed for nausea and vomiting.<sup>2</sup> Generally conventional medicines suppress symptoms rather than treating the underlying cause, and when taken during pregnancy does have a threat of various side effects on the developing fetus.

Homeopathy is controversial, science and has evoked scepticism.<sup>3,4</sup> The most serious criticisms related to homeopathy are the lack of understanding of mechanisms behind these remedies.<sup>5</sup> It is said that homeopathy works at the cellular level and therefore, researchers need high quality cells/model systems along with powerful techniques to prove the efficacy of homeopathy. To this end various researchers over the years have tried to ascertain the mechanism by which these ultra-diluted preparations (which usually lie beyond the Avogadro's limit) exert their effect. By using DNA microarray based transcriptomic analysis studies have revealed alterations in the gene expression of key signaling pathways.

Saha et al<sup>6</sup> showed that in the cervical cancer HeLa cells exposed to *Condurango* 30C and *Hydrastis canadensis* 30C there was a significant quantitative as well as qualitative difference in gene expression. Preethi et al also reported differential gene expression when Dalton's lymphoma tumor cells were treated with 200C potencies of *Ruta*, *Carcinosinum*, *Hydrastis* and *Thuja*.<sup>7</sup> In an elegant review. Bellavite et al have discussed research findings of the use of homeopathic remedies for various disease models. They have reported global alterations in gene expression in treated cells using DNA microarray.<sup>8</sup> These studies prove beyond a doubt that homeopathic remedies are not placebos but act by modulating gene expression.

Using extremely diluted dynamized dilutions (3C, 5C and 7C) of *Apis mellifica* in RPEW-1 prostrate cells, Bigagli and coworkers reported modulation of hundreds of genes using global gene profiling microarrays and carried out cluster analysis wherein certain groups of genes were up- or down-regulated with similar expression profiles among treatments; other genes showed opposite regulation profiles at low and high dilutions of *Apis mellifica*, suggesting a hormetic response.<sup>9</sup> Hence using alterations in gene expression as an endpoint to ascertain effect upon exposure, we treated embryonic stem cells with homeopathic remedies *Nux Vomica* and *Sepia*, which are routinely prescribed in early pregnancy.<sup>10</sup>

*Nux vomica* is the dried, ripe seed of *Nux Vomica L.* (family, Loganiaceae). *Nux Vomica* is given to counter symptoms of nausea and constipation during pregnancy.<sup>11</sup> *Sepia* is a product from the fresh ink of *Sepia officinalis* which is commonly known as cuttlefish. *Sepia* is prescribed to counteract morning sickness, constipation and headache during pregnancy.<sup>11</sup> The safe use of *Nux Vomica* and *Sepia* in pregnancy however is not well documented. Moreover, as these homeopathic remedies are over the counter drugs there is no direct regulatory control for their use. It is therefore, necessary to carry out research on these remedies to generate sufficient experimental data for their safe use in pregnancy.

Embryonic stem cell research contributes to a fundamental understanding of how an organism develops and

grows, and how tissues are maintained throughout adult life. The development of ES cell lines has provided researchers with the reliable tool to study developmental toxicity and birth defects.<sup>12,13</sup> The effects of conventional medicine taken during pregnancy have been studied using this *in vitro* EST model. Currently, there are no methods for testing developmental toxicity associated with homeopathy medicines to prove their safe use in pregnancy. In this study, various lineage specific genes were studied which could serve as biomarkers for specification of these three lineages to predict the safety of homeopathic remedies using mouse ES cells as the model system.

## Materials and methods

### Cell lines and culture conditions

The mouse ES cell line D3 was maintained at 37°C and 5% CO<sub>2</sub> and routinely passaged three times a week. ES cells were cultured in Dulbecco's modified Eagles Medium (DMEM) (Invitrogen), supplemented with 15% heat inactivated fetal bovine serum (FBS) (Sigma, USA), 2 mM glutamine (Invitrogen), 50 U/ml penicillin and 50 µg/ml streptomycin (Invitrogen), 1% non-essential amino acids (Invitrogen), 0.1 mM β-mercaptoethanol and 1000 U/ml LIF (leukemia inhibitory factor, ESGRO, Chemicon International Inc., Temecula, CA).

NIH 3T3 cells were procured from National Center for Cell Sciences, Pune. NIH 3T3 was maintained in complete medium, which was composed of DMEM (Invitrogen), supplemented with 10% FBS, 50 U penicillin/ml and 50 µg streptomycin/ml. When the cell were approximately 80–90% confluent, they were subcultured (2 times a week).

### Homeopathy remedies used for testing

The most common potency prescribed in pregnancy is 30C, as it is considered safe during first trimester in pregnancy by CCRH (Central Council for Research in Homeopathy, India).<sup>10,14</sup> The 30C potency of the homeopathic remedies *Nux Vomica* and *Sepia* were tested at concentrations of 1 µl/ml and 5 µl/ml. These remedies were obtained from Wilmar Schwabe, Germany. The concentration was selected on the basis of review of homeopathic database citing its use in *in vitro* studies.<sup>15</sup>

As homeopathic remedies are prepared in 90% ethanol,<sup>16</sup> there was a need to limit the maximum concentration of ethanol to below 0.5% v/v to negate the effect of the solvent.<sup>17</sup> The final concentration of ethanol was ≤ 0.5% in the all homeopathic remedies which were used as test concentrations. Ethanol at 0.5% concentration was used as the solvent control. For the positive control a stock of 5-fluorouracil (5-FU) at 1 mg/ml (Sigma) was prepared in PBS and sterile filtered through a 0.22 µm filter (Millipore). Aliquots were made and stored at –20°C. Further dilutions were made in DMEM to obtain a concentration range of 0.000625–1.78 µg/ml. PBS at a final concentration of 1% was used as the solvent control.

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