ABSTRACT

Homeopathic medicines have garnered much attention and debate in recent years. However, well documented studies are required for their potential effects and safety issues regarding their use in pregnancy. In this study, mouse embryonic stem (ES) cells were used as a model system and exposed to 30C potency of Nux Vomica and Sepia, which are medicines prescribed for the management of pregnancy related symptoms. Cytotoxicity studies were done using a modified ES cells testing (EST). The expression level of key genes and proteins expressed during differentiation was analyzed using real time polymerase chain reaction and immunocytochemistry, respectively. The results showed that homeopathic treatment led to slight modulations in the expression of certain lineage specific genes but this effect was not significant and showed normal differentiation as demonstrated by the expression of α/β MHC and α-actinin proteins in the differentiated ES cells. This study for the first time showed the use of ES cells in the developmental toxicity testing of homeopathy and the results obtained further support the use of these medicines in pregnancy.
3.1 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 [1,2]. The popularity of homeopathy amongst the believers in this system of medicine is not based on hearsay but on rational, systematic studies and observation of the effects of the remedies on people. Moreover, 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. Being incalculably small in concentration, these medicines have not shown to induce any adverse effects even when taken for extended periods and can therefore be safely given even to drug sensitive group like pregnant women, babies and elders. Conventional medicine generally suppresses 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 balances the whole system and thus allowing the body to heal itself. Since homeopathy works with the body's natural defense system, it is advocated by the homeopaths that homeopathic medicines pose no harmful effect on developing embryo.

The foundation for the homeopathic system of medicine was systematized by the German physician Samuel Hahnemann in 1796. The main principle behind the action of homeopathy is the Similarity (or Similia) Principle: ‘Similia similibus curentur’ (‘Let like be cured by like’), stated by Samuel Hahnemann [3]. Simply put, this means that a substance which has potential of causing disease in healthy organisms can be used as medicines for treatment of similar patterns of disorder experienced by patients.

A second principle of homeopathy is individualization of treatment for the patient. The properties of the chosen medicine must be as similar as likely to the symptoms of the disease in the patient. This most compatible match is called the ‘simillimum’. Resemblance may be at the ‘whole person’ level, counting the symptoms and signs of the disease, the patient’s physical built, personality, temperament and genetic predisposition. This great level of individualization is however not always needed: ‘similarity’ may be at a more particular, local level, especially in the curing of severe conditions [3].

A third principle of homeopathy is the use of the minimum dose. The dilutions used in treatment ranges from those which are similar in concentration to some conventional medicines
to very high dilutions containing no material trace of the starting substance. Vigorous shaking of the solution along with impact or ‘elastic collision’ (known as ‘succussion’) is an established manufacturing process and is a fundamental element in the production of homeopathic medicines [4, 5].

Dilutions are designated by numbers and letters, for example, 30X or 30C. These designated letters and numbers indicate the potency of the homeopathic remedy. ‘X’ potency is made by taking one drop of a natural material substance (like ink from squid-\textit{Sepia}) and diluting it in 10 drops of water/alcohol (X is 10 potency). ‘C’ potency is taking the same substance and diluting it in 100 drops of water/alcohol (C is 100\textsuperscript{th} potency). Then one drop is taken from that X or C potency after succussion and diluted in another 10 or 100 drops, respectively. Each stage produces the next potency as in 10X to 11X or 12C to 13C. Potencies can be diluted and succussed thousands of times to produce M potency (1000), 10M, 50M, CM (100M) and MM (1000M). It should be noted that these high potencies are not to be used lightly and should be given by experienced homeopaths. The most common potencies used in pregnancy are 30C and 200C as they are considered safe during first trimester in pregnancy by CCRH,(Central Council for Research in Homeopathy), India [6].

Homeopathy is a controversial science. The elucidation of how homeopathic medicines work, has evoked the cynicism from the scientific community[7, 8]. The most serious criticisms related to homeopathy are the lack of transparency of mechanisms behind these remedies [9, 10]. It is said that homeopathy works at the cellular level as all the biological and physiological activities occur at the cellular level. Researchers need high quality cells/model systems to prove the efficiency of homeopathy and herein lies the dilemma of homeopathy’s potential because of the lack of scientific data [11]. Thus, the present work was done to suggest a model system to study the effects of homeopathic remedies taken during pregnancy using ES cells as the model which mimics \textit{in vivo} development of the embryo. The two homeopathic remedies \textit{Nux Vomica} and \textit{Sepia} under study were chosen as they are routinely prescribed in early pregnancy to counteract many of the symptoms encountered during this stage [12].

\textit{Nux vomica} is the dried, ripe seed of \textit{Nux Vomica} \textit{L.} (Family, Loganiaceae), a native tree of Burma, China, eastern India, Thailand, and northern Australia\cite{13}. As homeopathic remedy, \textit{Nux Vomica} is given for allergies, back pain, colds, constipation, digestive problems, emotional
stress, flu, hangovers, headaches, hemorrhoids, and menstrual problems. *Nux vomica* is a popular homeopathic remedy used in pregnancy and is given for nausea. Although *Nux Vomica* appeared as a treatment in 19th Century medical publications, there is a very little documentation on its therapeutic effectiveness in today's standard medical journals. *Nux Vomica* is a common homeopathic remedy and is given to counter symptoms of nausea and constipation during pregnancy. It is therefore, necessary to carry out research on this remedy to generate sufficient experimental data for its safe use in pregnancy [13-15].

*Sepia* is a product from the fresh ink of *Sepia officinalis* which is commonly known as cuttlefish. When this cuttlefish sense danger it releases out ink which serves for both defensive and aggressive purposes. This ink has been used as a source for making the homeopathy remedy *Sepia* [15-17]. *Sepia* is a popular homeopathy remedy prescribed to counteract morning sickness, constipation and headache during pregnancy. But the safety use of *Sepia* in pregnancy is not well documented. Moreover, these homeopathic remedies are regulated as over the counter drugs.

ES cells are the source of all organs and tissues of the body. Being pluripotent in nature, they have the potential to differentiate into all cells found in the body. In their undifferentiated state, they can be maintained in indefinitely and with capable of differentiation *in vitro* - represent an unlimited source of somatic cells. ES cells research contributes to a fundamental understanding of how organisms develop and grow, 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 [18]. 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 of homeopathy medicines to prove their safety 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.
3.2 MATERIALS AND METHODS

3.2.1 Cell lines and culture conditions

The mouse ES cell line D3 was cultured at 37°C and 5% CO₂ and routinely passaged three times a week. ES cells were cultured in Dulbecco’s modified Eagles Medium (Invitrogen), supplemented with 15% heat inactivated fetal bovine serum (FBS), 2mM glutamine (Invitrogen), 50U/ml penicillin and 50μg/ml streptomycin (Invitrogen), 1% non-essential amino acids (Invitrogen), 0.1mM β-mercaptoethanol and 1000U/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 Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen), supplemented with 10% fetal bovine serum (FBS) (Sigma, USA), 50U/ml penicillin and 50µg/ml streptomycin (Gibco). When the cell were approximately 80–90% confluent, they were subcultured (2 times a week).

3.2.2 Homeopathy remedies used for testing

The 30C potency of the homeopathic remedies Nux Vomica and Sepia were tested at concentrations of 1µl/ml and 5µl/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 pregnancy. As homeopathic remedies are prepared in 90% ethanol, it was needed to limit the maximum concentration of ethanol to below 0.5% v/v so as to negate the effect of the solvent. So, the final concentration of ethanol was ≤ 0.5% in the test concentrations and thus ethanol at 0.5% concentration was used as a solvent control.

3.2.3 MTT assay

Cytotoxicity determination of the homeopathic remedies was done on the basis of MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide) assay according to the modified shortened EST protocol using NIH 3T3 fibroblast and D3 ES cells, as previously described [18, 19]. In brief, trypsinized cells were counted using hemocytometer and seeded in each well of 96 well plates at density of 20,000 cells/50 µl media. After 4 hours of incubation, the cells were treated with 150µl of culture media containing homeopathic remedies at concentration of 1µl/ml and 5µl/ml. The media was replaced on day 3 with fresh media containing the same.
concentrations of the remedies. Cytotoxicity was assessed using the MTT on day 5. Following this, 20µl of MTT solution (5mg/ml in phosphate buffer saline) was added to each well containing the cells and incubated for 4 hours at 37°C. Then the media was replaced with 150µl of DMSO and incubated for next 20 minutes. Absorbance of the formazan products due to the viable cells was measured at a wavelength of 570nm with reference wavelength 630nm in microplate reader (BIO-RAD model 680) and the concentration-response curve was obtained. The assay was performed four times in triplicates. The cytotoxicity was calculated as per the formula given below:

\[
\% \text{ Cytotoxicity} = \frac{\text{O.D. of solvent control} - \text{O.D. of test sample}}{\text{O.D. of solvent control}} \times 100
\]

### 3.2.4 Flow cytometry analysis for cell viability

Flow cytometry was performed using an Accuri C6 flow cytometer (BD Sciences) to analyze cell viability using propidium iodide (PI) staining dye. For PI cell viability analysis, 1.5x10^5 cells were seeded in 6 well plates. The seeding day was considered as day zero. After 24 hours, old media was replaced with fresh media along with homeopathic remedies (Nux Vomica and Sepia) in different concentrations (1µl/ml and 5µl/ml) and kept for a period of 3 days. At day 3, the media was again replaced with fresh media containing the homeopathic remedies at the specific concentrations. On day 5, cells were trypsinized and the pellet was collected after centrifugation at 80 g for 5 minutes and PI (5µg/ml) treatment was given for 30 minutes. This was followed by the analysis of live and dead cell population in Accuri C6 flow cytometer. PI is excited at 488nm and emits at a maximum wavelength of 617nm. The experiment was done three times in triplicates.

### 3.2.5 Differentiation of ES cells

To analyze the effects of homeopathic remedies on differentiation of ES cells, two different differentiation protocols were performed: the hanging drop method and the monolayer differentiation method. The choice of method was dependent on the assay that had to be performed.
3.2.5.1 EB differentiation

The EB assay was selected for the differentiation experiments as EBs mirror the early steps of embryogenesis for both mouse and human ES cells differentiation protocols [20, 21]. Differentiation was carried out in hanging drops according to a modified method done by Heuer et al [21]. In brief, a drop of 20 μl from ES cell suspension (5 x 10⁴ cells/ml) was placed onto the inner side of the lid of a Petri dish (Corning) filled with phosphate buffered saline (PBS) (Himedia) and then incubated at 37°C with 5% CO₂. After culturing for 3 days, the formed aggregates (EBs) were transferred into bacteriological petri dishes (non-treated). At day 5, EBs were plated separately onto gelatin coated coverslips in 6 well plates (Thermo Scientific) for ICC analysis at day 10.

3.2.5.2 Monolayer differentiation

The cells were seeded at density of 5 x 10⁴/ml in 6 well plates (Thermo scientific) in Dulbecco’s modified Eagles Medium (Invitrogen), supplemented with 15% heat inactivated fetal bovine serum (FBS), 2mM glutamine (Invitrogen), 50U/ml penicillin and 50μg/ml streptomycin (Invitrogen), 1% non-essential amino acids (Invitrogen), 0.1mM β-mercaptoethanol without LIF (leukemia inhibitory factor). The RNA was isolated on day 5.

3.2.6 Immunocytochemistry (ICC)

D3 (EBs) were obtained from hanging drop methodology and transferred onto gelatin coated coverslips in 12 well plate at day 5. After culture for next 5 days, the EBs were fixed in paraformeldehyde solution (4% in PBS) for 30 minutes at room temperature. After a PBS wash, EBs were permeabilized with Triton X-100 (0.25% in PBS) added for 15 minutes followed by washing with wash buffer [1% bovine serum albumin (BSA) in PBS]. This was followed by incubation for 60 minutes with 5% BSA in PBS at room temperature to block non-specific binding and again washed with wash buffer. EBs were then incubated with primary antibodies against α/β MHC (Abcam) and α-actinin (Sigma) which were diluted at 1:250 and 1:200, respectively, and kept overnight at 4°C. After washing with wash buffer, cells were incubated with secondary antibody: Fluorescein-5-isothiocyanate (FITC)-conjugated rabbit anti-mouse (Sigma) diluted in the ratio 1:500 and again washed with wash buffer. The EBs were viewed under the Nikon eclipse Ti microscope at 10X magnification using the appropriate filters.
excitation at 530nm for FITC staining. The ICC experiment was performed three times in triplicates.

3.2.7 RNA isolation, cDNA synthesis and quantitative real time PCR (qRT-PCR) for analyses of gene markers associated with differentiation

The RNA was isolated with RNeasy Mini Kit (Qiagen) and included DNA digestion. The concentration and quality of RNA was measured with a Nano drop 2000 spectrophotometer (Thermo Scientific). Synthesis of cDNA from RNA was carried out by using an oligo dT (15) primer in the presence of M-MULV Reverse Transcriptase (Genetix). PCR was performed with 0.5µg c-DNA (cDNA) of each sample using gene specific primers in order to determine the expression level for target gene. qRT-PCR was performed on CFX-96 real time PCR (Bio-Rad laboratories) using SYBR Green real time PCR dye (Bio-Rad laboratories). The conditions were: initial denaturation at 93°C for 4 minutes, followed by 39 cycles each of denaturation (95°C for 15 s seconds), annealing (54.5-60.2°C for 20 seconds) and extension (72°C for one minute). The relative quantitative expressions of lineage specific markers were calculated after normalization against GAPDH, a housekeeping gene. The present study analysed a total of seven gene markers which are associated with the formation of three different lineages. The gene markers associated with the formation of mesodermal lineage included Brachyury, Flk-1, Nkx2.5 and α/β MHC while (Afp) Alpha fetoprotein was selected due to its association with the development of endoderm. For studying the expression of ectodermal lineage, nestin (Nes) and Neurofilament 200 kDa (ND200) gene markers were analyzed. The details of the primers with annealing temperatures are provided in the Table 3.1. The gene expression analysis was performed three times in triplicates.
### Table 3.1: Detail of the primers used in this study

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer sequence (Forward primer-FP and Reverse primer-RP)</th>
<th>Annealing temperatures (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>FP 5’-GCACAGTCAAGGCCGAGAAT-3' RP 5’-GCCTTCTCCATGGTGTTGAA-3’</td>
<td>58.5</td>
</tr>
<tr>
<td>Afp</td>
<td>FP 5’-GCTGCAAAGCTGACAACAAG-3’ RP 5’-GGTTGTTGCCTGGAGGTTC-3’</td>
<td>58.7</td>
</tr>
<tr>
<td>Flk-1</td>
<td>FP 5’-CAGCTTCCAAGTGCTAAG-3’ RP 5’-CAGAGCAACACCAGCAGAA-3’</td>
<td>54.5</td>
</tr>
<tr>
<td>Nes</td>
<td>FP 5’-GCTTTTCTGACCACCAGCTG-3’ RP 5’-GGCAAGGGGAAGAGGA-3’</td>
<td>60.2</td>
</tr>
<tr>
<td>ND 200</td>
<td>FP 5’-TGGACATTTGAGATGCCC-3’ RP 5’-GAGAGGAGGACTCGGACCA-3’</td>
<td>62.4</td>
</tr>
<tr>
<td>Nkx2.5</td>
<td>FP 5’-CAAGTGCTTCTCTGCTTTC-3’ RP 5’-GGCTTTGTCCAGCTCCACT-3’</td>
<td>56.5</td>
</tr>
<tr>
<td>α/β MHC</td>
<td>FP 5’-ACCTGCAAAGTTCCGCAAG-3’ RP 5’-CTTGTGACCTGGAGCTCGG-3’</td>
<td>58.5</td>
</tr>
<tr>
<td>Brachyury</td>
<td>FP 5’-TTCTTTGGCATCAAGGAAGG-3’ RP 5’-TCCCGA GACCCAGTTCA-3’</td>
<td>57.0</td>
</tr>
</tbody>
</table>

#### 3.2.8 Statistical Analyses

The cytotoxicity data was obtained by calculating the mean±SEM (standard error mean) from four individual experiments done in triplicates. The cytotoxic effects of homeopathic remedies were statistically analyzed with one-way ANOVA followed by Tukey test using
The ICC experiment was performed three times in triplicates. The effect of these remedies on the expression levels of the lineage specific markers were analyzed using the \(2^{-\Delta\Delta\text{Ct}}\) method. The gene expression analysis was obtained by calculating the mean ± SD (standard deviation) from 3 individual experiments done in triplicates. The statistical analysis was done using two-way ANOVA and followed by a Bonferroni test using GraphPad Prism software version 6.0.

### 3.3 RESULTS

#### 3.3.1 Determination of the cytotoxicity of homeopathy remedies using ES cells (D3 cells) and adult fibroblast cells (3T3 cells)

For cytotoxicity analysis, MTT assay according to a modified EST model was performed to study the cytotoxicity effects on D3 and 3T3, cells which represented embryonic and adult cells, respectively. In both cell types, the homeopathic remedies namely *Nux Vomica* and *Sepia*, were tested at their different concentrations.

Sensitivity of D3 cells showed varied responses upon treatment with the homeopathic remedies. Cytotoxicity in D3 cells treated with 30C potency of *Nux Vomica* showed a killing of 15.47±2.5% at 1µl/ml concentration and 20.36±4% at 5µl/ml concentration while cytotoxicity in D3 cells treated with 30C potency of *Sepia* showed a killing of 17.71±3.0% at 1µl/ml concentration and 18.73±4.0% at 5µl/ml concentration, with respect to the solvent control of D3 cells (Figure 3.1a). The percent cytotoxicity of D3 cells exposed to homeopathic remedies was found to be non significant with respect to the solvent control. Although slightly higher cytotoxicity was observed at the higher concentration (5µl/ml) as compared to 1µl/ml, however, this was a non significant difference in the observed cytotoxicity in the D3 treated cells which had been exposed to the two different concentrations of both *Sepia* and *Nux Vomica* used in this study. The statistical analysis was done by using one-way ANOVA followed by Tukey test.

3T3 cells also showed increased cytotoxicity with increasing concentration of 30C potency of *Nux Vomica*. i.e. 4.70±1.2% at 1µl/ml concentration and 15.9±2.7% at 5µl/ml concentration. Similarly, upon treatment with 30C potency of *Sepia*, 3T3 cells showed increased cytotoxicity with increase in concentration i.e. 8.07±1.9% at 1µl/ml concentration and 13.77±3.50% at 5µl/ml concentration, with respect to the solvent control of 3T3 cells (Figure
The statistical analysis which was done by using one-way ANOVA followed by Tukey test revealed that percent cytotoxicity observed in the 3T3 cells treated with the homeopathic remedies was nonsignificant with respect to both the solvent control as well as between the two concentrations under study and this held true for both Sepia and Nux Vomica. Although marginal cytotoxicity was observed upon treatment of ES and 3T3 cells with the homeopathy remedies, however, these results were not statistically significant. Although, the sensitivity of homeopathic remedies towards D3 was more as compared to 3T3 cells as evidenced by increased cell death observed in the D3 cells. The cytotoxicity data was represented as concentration–response curves as shown in Figure 3.1.

**Figure 3.1:** Concentration–response curves obtained for homeopathic remedies. Cytotoxicity was assessed by the MTT assay in (a) undifferentiated D3 cells and (b) differentiated 3T3 cells. Bar graphs show means ± SEM. All values are average of four experiments done in triplicates.
3.3.2 Analysis of cell viability using flow cytometry

The flow cytometry data revealed that the homeopathic remedies had no effect on cell viability of both cell types. It was observed that D3 cells after exposure to *Nux Vomica* 30C potency at 1µl/ml concentration had 81.7% live cell population and at 5µl/ml concentration, the live population was 85.9%. This was not statistically significant as compared to the control (untreated D3 cells which had 86.6% and solvent control had 86.8% live cell population). Treatment of 30C potency of *Sepia* on the D3 cells followed a similar trend and was also found to be non significant as compared to the control i.e. 1µl/ml concentration resulted in 85.9% of live population and 5µl/ml concentration resulted in 84.3% of live population (Figure 3.2). 3T3 cells also showed a similar trend as the data revealed that treatment of 30C potency of *Nux Vomica* had 78.1% and 70.1% live cell population upon treatment with 1µl/ml and 5µl/ml concentration, respectively. In contrast to this, the control cells had 84.4% and solvent control had 82.5% of live cell population.

Similarly, 30C potency of *Sepia* also showed no sensitivity on 3T3 cells as compared to the controls (untreated 3T3 cells which had 84.4% and solvent control had 82.5% live cell population) i.e. they exhibited 73.8% live population upon treatment with 1µl/ml concentration and 75.8% live cell population upon treatment with 5µl/ml concentration. These results further confirmed that the both the cells types were not harmed by homeopathic remedies because the effects were similar to controls and moreover were not significantly different (Figure 3.3).
Figure 3.2: Flow cytometry analysis showing the cell viability of D3 cells after treatment with homeopathic remedies on day 5. (a) untreated control D3 cells; (b) Solvent control treated D3 cells; (c) D3 cells treated with 1µl/ml concentration of Nux Vomica 30C potency; (d) D3 cells treated with 5µl/ml concentration of Nux Vomica 30C potency; (e) D3 cells treated with 1µl/ml concentration of Sepia 30C potency; (f) D3 cells treated with 5µl/ml concentration of Sepia 30C potency.
Figure 3.3: Flow cytometry analysis showing the cell viability of 3T3 cells after treatment with homeopathic remedies on day 5. (a) untreated control of 3T3 cells; (b) solvent control treated 3T3 cells; (c) 3T3 cells treated with 1µl/ml concentration of Nux Vomica 30C potency; (d) 3T3 cells treated with 5µl/ml concentration of Nux Vomica30C potency; (e) 3T3 cells treated with 1µl/ml concentration of Sepia30C potency; (f) 3T3 cells treated with 5µl/ml concentration of Sepia 30C potency.
3.3.3 Immunocytochemistry of mesodermal markers of EBs exposed to homeopathic remedies

ICC analysis of α/β MHC and α-actinin revealed that remedies had no effect on differentiation of the EBs when tested at the highest concentration under study i.e. 5µl/ml concentration of 30C potency. On observing Figure 3.4, untreated control and solvent control EBs (without homeopathic treatment) showed expression of α/β MHC as well as α-actinin which were comparable to that of the treated EBs (5µl/ml concentration of homeopathic remedies). Treatment with 5µl/ml concentration of 30C potency Nux Vomica and Sepia represented EBs stained with antibodies against α/β MHC and α-actinin, which showed normal growth and differentiation. In addition, there was no variation in the size of the EBs as compared to solvent control (Figure 3.4).
Figure 3.4: ICC analysis of EBs stained with cardiomyocytes differentiation marker α/β MHC and α-actinin after treatment with homeopathic remedies at day 10. Magnification 10X. Bars: 100μm. The experiment was performed three times in triplicates.

3.3.4 Expression of marker genes of differentiation of ES cells exposed to Nux Vomica (30C potency)

To compare the changes in the expression of genes responsible for lineage commitment, the untreated and treated ES cells were subjected to monolayer differentiation. The expression level of genes considered as the markers of differentiation during embryogenesis was analyzed to identify the changes caused by exposing the ES cells to homeopathic remedies Nux Vomica and Sepia (potency 30C).
It was found that the cells exposed to Nux Vomica 30C potency (1µl/ml and 5µl/ml, concentrations) for 5 days down-regulated the expression of early mesodermal marker, Brachyury to 0.52±0.11 fold and 0.50±0.08 fold, respectively. The Flk-1 gene which is also an early mesodermal marker was also down-regulated to the same extent i.e. 0.55±0.07 fold for 1µl/ml concentration and 0.38±0.05 at concentration of 5µl/ml. The markers for cardiomyocytes *i.e.* α/β MHC and Nkx2.5 showed negligible down-regulation in expression upon treatment with 30C potency of Nux Vomica. 1µl/ml concentration of 30C potency Nux Vomica resulted in the decreased expression of α/β MHC to 0.92±0.05 fold and Nkx2.5 to 0.82±0.11 fold. Similarly, 5µl/ml concentration also resulted in the down-regulation of α/β MHC expression to 0.55±0.07 fold and Nkx2.5 to 0.53±0.09 fold. All the changes observed were not significant with respect to the solvent control (Figure 3.5a).

Afp which represents the endodermal lineage was also down-regulated to 0.61±0.14 fold in case of 1µl/ml concentration and 0.53±0.05 fold after exposure to 5µl/ml concentration (Figure 3.5b).

Ectodermal markers, Nes and ND200 were also found to be down-regulated to the same extent *i.e.* 0.63±0.07 fold at 1µl/ml concentration and at 5µl/ml concentration, the change was 0.60±0.09 fold (Nes) and 0.32±0.05 fold (ND200) (Figure 3.5c).

Although down-regulation for all the genes of the 3 lineages was observed but this effect was not significant.
Figure 3.5: Gene expression analysis of three lineages of ES cells exposed to homeopathic remedy Nux Vomica 30C (a) mesodermal; (b) endodermal; (c) ectodermal. Expression level was normalized to housekeeping gene i.e. GAPDH. The y-axis represents the fold changes in the expression of the gene under study. The data was analyzed by using the $2^{-\Delta\Delta Ct}$ method. Bar graphs show means ± SD (n = 3). Significance between treated cells and untreated cells was calculated by applying two-way ANOVA.

### 3.3.5 Expression of markers genes of ES cells differentiation after exposure to Sepia 30C potency remedy

*Sepia*, homeopathic remedy at 30C potency decreased the expression of genes in concentration-dependent manner. Brachyury and Flk-1, genes expression level after exposure to 1µl/ml concentration was reduced to 0.52±0.06 and 0.82±0.12,fold, respectively. After exposure to 5µl/ml concentration, the expression level was found to be 0.41±0.07 and 0.66±0.09 fold, respectively. The cardiac marker, α/β MHC showed 0.74±0.11 fold and Nkx2.5 showed 0.72±0.1 fold expression after exposure to 1µl/ml concentration of 30C potency and 0.61±0.09 and 0.60±0.09, fold after exposure to 5µl/ml concentration of 30C potency (Figure 3.6a). Similarly, the Afp was also decreased and showed 0.65±0.14 and 0.48±0.05,fold change with 1µl/ml and
5µl/ml, concentration as compared to normal expression (Figure 3.6b). The ectodermal markers were also influenced by the 30C potency Sepia (Figure 3.6c). The Nes expression was 0.58±0.08 fold and ND200 expression was 0.69±0.05 fold, after exposure to 1µl/ml concentration. The expression of Nes at 5µl/ml concentration was 0.55±0.07 fold. The expression of ND200 was found to be 0.53±0.08 fold after exposure to 5µl/ml concentration.

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**Figure 3.6:** Gene expression analysis of three lineages of ES cells exposed to homeopathic remedy Sepia 30C (a) mesodermal; (b) endodermal; (c) ectodermal. Expression level was normalized to housekeeping gene i.e. GAPDH. The y-axis represents the fold changes in the expression of the gene under study. The data was analyzed by using the $2^{-\Delta\Delta C_t}$ method. Bar graphs show means ± SD (n = 3). Significance between treated cells and untreated cells was calculated by applying two-way ANOVA.

The results from the gene expression analysis during the differentiation of mouse ES cells provided new insights to the effects of this homeopathic remedy. It is clear from these results
that remedies which were advocated as safe during pregnancy have been validated by experimental data.

3.4 DISCUSSION

Over the last decade, the use of homeopathy remedies has grown exponentially. However, knowledge regarding harmful effects of these remedies by users is limited and available data is conflicting. This is of particular concern in pregnancy, where need of considering their potential effects on fetal development and drug interactions are of extreme importance. The mechanism of action of these remedies and factors related to their use in pregnancy need extensive studies for safety assessment during this sensitive stage of life. This research work provides the first step in evaluating and providing scientific evidences for effects of homeopathic remedies during embryogenesis.

In order to know the causes of congenital abnormalities due to drug intake, ES cell has been the first choice as a model system, to identify toxicities that may potentially be encountered in the embryos. Therefore, in this study the potential of ES cells was used in exploring the effects of homeopathic remedies taken during pregnancy.

Homeopathic remedies are commonly used for common problems in pregnancy e.g. nausea, constipation, indigestion by a large section of society in Asia and certain regions of Europe [23, 24]. The focus of this study was to evaluate the possible effects of the 30C potency on the differentiating ES cells at an early stage of the developmental process which mimics the growing embryo. In this study, the cytotoxic effects of Nux Vomica and Sepia homeopathic remedies on both adult cells and ES cells were analyzed. The analysis of key genes which mark the onset of differentiation of the 3 lineages was studied using qRT-PCR analysis which revealed nonsignificant alterations in the genes upon treatment with these remedies. These results will help in convincing the skeptics that these medicines prescribed during pregnancy are indeed safe and do not contribute to any developmental toxicity at the potency of 30C. These results are in conformity with other well documented studies with regard to the safety assessment of homeopathic remedies on normal cell lines [25-27].

*Nux Vomica* is a popular homeopathic remedy prescribed for symptoms such as morning sickness and constipation, which are frequently encountered during pregnancy. In this study, it was observed that *Nux Vomica* had a marginal effect on cell viability and gene expression. It was
seen that the early mesodermal biomarkers, Brachyury and Flk-1 were not significantly sensitive to 30C potency and this was true for the cardiac markers α/β MHC and Nkx2.5 as well which showed similar sensitivity to 30C potency. The normal expression of α/β MHC and α-actinin which are key proteins involved in the differentiation towards cardiomyocytes and are required for the proper development and functioning of heart, were also documented. These effects on gene and protein expression upon treatment with 2 different concentrations revealed that although changes were not significant as compared to controls, however, the remedies did act in a concentration-dependent manner leading to the effects on expression of genes and that embryonic cells were more sensitive to the treatment as compared to adult cells.

Sepia is derived from ink of the cuttlefish (is the pigment Melanin), which is comprised of sulphur, calcium, and magnesium, among other compounds and it appears bilious like a dark stain[15, 16]. The effect of Sepia 30C potency on differentiation of key lineage genes was not significant. For both adult as well as EScells, the results followed a similar trend as that of Nux Vomica exposure. The non cytotoxic nature of the homeopathic medicines was reiterated by the fact that the ES cells which were allowed to aggregate in the form of EBs showed a size comparable to untreated ES control cells and differentiated to express key proteins α/β MHC and α-actinin, that are present in cardiomyocytes. This demonstrated no impact on the differentiation capability of the ES cells which were exposed to either Nux Vomica or Sepia.

In this study, a key question was raised whether the homeopathic remedies were having effects on the growing embryo which could lead to abnormalities? In order to study this, a panel of genes representing the three lineages was chosen. The rationale was that if there were significant changes in the expression of these key genes then that would indicate adverse effects to the embryo. The choice of the genes to be assessed was based upon previous studies in which various researchers had used these genes to demonstrate the developmental toxicity upon exposure to chemical compounds [22, 28, 29]. In the mouse ES cell-based differentiation model, Brachyury is known determinant of meso-endoderm lineage, and cells which are positive in Brachyury expression are able to differentiate into mesodermal and definitive endodermal lineages[30]. Flk-1 gene has biphasic potential in embryogenesis in which early expression leads to hematopoietic lineages and later on the expression is positive for cardiovascular progenitor cell potential [31]. In embryogenesis, Flk-1 is expressed by the primitive endoderm, embryonic angioblasts and in the blood islands as well as in angiogenic vessels. The aimed disruption of
gene Flk-1 ended in mice lethality at E8.5-E9.5 due to lack of development of the blood islands, embryonic vasculature and hematopoietic cells [32, 33]. The expression of α/β MHC in mammalian hearts can be expressed in two isoforms; α-MHC is solely expressed in cardiac tissue, whereas β-MHC expression is observed in both cardiac and slow skeletal muscle. The expression of α/β MHC plays an important function in the development of contraction and adaptation in cardiac muscle [34, 35]. The normal expression of this α/β MHC gene was clearly observed in ES cells after treatment with homeopathic remedies. Nkx2.5 which is a dynamic transcription factor functions in various aspects of early cardiac development leading to specification and proliferation of cardiac progenitor cells and its expression is constant during development and in adult life [36, 37]. This homeobox gene, Nkx2.5 [38] is the most primitive known marker of mammalian cardiac development. It was reported that targeted disruption of murine Nkx2.5 resulted in embryonic lethality and cardiac arrest at the linear heart tube stage prior to looping [39]. In this study, homeopathic remedies did not alter the expression of mesodermal genes under study.

Afpl gene expression leads to endoderm specification during embryogenesis [40]. Its expression is used extensively as biomarker of endoderm lineage. Its sensitivity is also used for studying embryotoxicity of drugs [41]. Altered levels of Afpl which are not within normal limits in pregnancy, have been reported for multitude of congenital malformations of the fetus [42-44]. Although, this study did observe that the expression of Afpl was decreased, this was not significantly and therefore, cannot be linked to the developmental effects of homeopathic remedies.

It has been reported that as neurogenesis proceeds, Nes (neural stem cell marker) is replaced by specific intermediate filaments [45, 46]. From this study, it was observed that homeopathy remedies could affect mouse ES cells but as the effects were non significant, they probably do not contribute to the developmental toxic effects on developing embryos.

With appropriate toxicity data sets, acceptable exposure levels and actual safety of prescription and nonprescription drugs as well as environmental chemicals could be established for individuals that are more vulnerable to chemical exposure, such as pregnant women and their unborn children. To reduce the spending of live animals, an assortment of in vitro assays has been proposed. By using ES cells and adult cells in testing system, differential effects to
embryonic and adult tissues can be assessed. Homeopathy has been regarded as an alternative system of medicine and its entry into mainstream conventional practice has been restricted owing to limited experimental data regarding safety and efficacy.

In conclusion, this study proposes that the expression of different lineage biomarker genes can be useful in examining the side effects of homeopathy which is an important but neglected issue in pregnancy. Implementing analysis of the expression of these biomarkers of differentiation for risk management of homeopathy use for a select section of users who are more susceptible to any drug exposure, such as pregnant women and their unborn children can go a long way in not only ensuring safety but would also lead to the promotion of homeopathic medicines to control various symptoms prevalent during pregnancy.
REFERENCES


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