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Monoisoamyl dimercaptosuccinic acid abrogates arsenic-induced developmental toxicity in human embryonic stem cell-derived embryoid bodies: Comparison with *in vivo* studies

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ABSTRACT

The ability of human embryonic stem (ES) cells to differentiate into the three germ layers has proposed its application in studying human developmental toxicity *in vitro*. In the current study we investigated if the prompted application could be utilized to evaluate the efficacy of a newly developed arsenic antidote, monoisoamyl dimercaptosuccinic acid (MiADMSA) against arsenic (III) and if the results obtained *in vitro* were in concordance with the animal model for studying developmental toxicity. On the basis of real time PCR (qRT-PCR) and cytotoxicity analysis of human embryoid bodies (EBs), we observed that arsenic (III) caused a significant down regulation of gene expression in all the three germ layers, which could be correlated with high mortality, visceral and skeletal defects in pups. Reversal of arsenic-induced dysfunctioning could be observed with concomitant treatment of MiADMSA *in vitro* and *in vivo*, indicating ES–EB model could provide toxicity information similar to *in vivo* model. IR spectroscopy further suggested that MiADMSA bind to arsenic to form adduct, which prevents arsenic from exerting its toxic effect in both models. To our knowledge this study provides first experimental evidence suggesting human ES cells could be utilized in studying the efficacy of drugs in a comparable manner with animal models. We conclude that the ES–EB model seems to be an effective, faster, cost effective method for predicting efficacy of a drug.

1. Introduction

Arsenic, a ubiquitously present metalloid exists in several different chemical and oxidation states (−3, 0, +3, +5), causing acute and chronic adverse health effects [1]. Exposure to arsenic may occur from natural or industrial sources. Acute exposure due to accidental uptake or intentional administration (in cases of suicidal or homicidal attempts) is rare and may result in gastrointestinal discomfort, vomiting, coma and sometimes even death. However, it is chronic poisoning which is of primary concern to environmental toxicologists, since it is widespread and insidious in nature. As many as 200 million people worldwide are at a risk, out of which 112 million are residing in the Indo–Bangladesh region alone and are suspected to be suffering from chronic arsenic poisoning through drinking water [2–4] containing levels more than the prescribed WHO limit of 50 μg/L [5]. Chronic exposure of arsenic causes a wide range of toxic effects involving multiple organ systems of the body; one of the important signs of chronic arsenic toxicity in humans is occurrence of skin lesions, characterized by hyper-pigmentation, hyperkeratosis and hypo-pigmentation [6,7].

Chelation therapy with 2,3-dimercaprol (BAL: British Anti-Lewisite) has been the main stay for arsenicosis but with the serious side effect of BAL, meso 2,3-dimercaptosuccinic acid (DMSA) has been tried in animals [8,9] and few cases of human arsenicosis [10] with beneficial effects. However, in a double blind, randomized controlled trial study conducted on patients from arsenic affected West Bengal (India) regions with oral administration of DMSA suggested that it was not effective in producing any clinical or biochemical benefits or any histo-pathological improvements of skin lesions [11]. In order to achieve optimal effect of chelation, large number of esters of DMSA were synthesized and tried against chronic arsenic intoxication in animal models [8,12–14]. Out of these esters, mono isoamyl derivative of DMSA (MiADMSA) has shown the maximum efficacy and better clinical recoveries in animal studies with arsenic and lead [4,15,16].

Abbreviations: MiADMSA, monoisoamy dimercaptosuccinic acid; DMSA, meso 2,3-dimercaptosuccinic acid; ES, human embryonic stem; HFF, human foreskin fibroblast; hES, human embryonic stem cell; hEB, human embryoid bodies.

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Although, animal models have invariably been extrapolated for humans but due to interspecies variations there may be variations like the thalidomide disaster [17,18]. In last one decade the knowledge of human developmental biology has been extended to the next level with the establishment of first human embryonic stem (ES) cell line by Thomson et al. [19]. The inherent property of human ES cells from the three germ layers via the embryoid body (EB) formation has added better insight in understanding human developmental biology *in vitro*. Human ES cells offer several important advantages over traditional cell based systems like primary or immortalized cultures. This stems from the fact that if maintained under optimized culture conditions they remain genetically normal and can be propagated indefinitely in culture, thereby providing a constant supply of cells in drug testing [20]. Human ES cell lines may therefore, prove clinically more useful and less time consuming in drug development for various human diseases. McNeish [20] also highlighted the importance and utility of human ES cells in the drug discovery process. Utilization of mouse ES cells for detection of the embryotoxic potential is already well established and validated by the ECVAM (European Centre for Validation of Alternative Methods) [21,22]. Recently, in a proof of concept study, we and others reported human ES cell derived EB model could be used to detect developmental toxicity more sensitively of known compounds and could be classified as non-, weakly and strongly embryo-toxic compounds based on cytotoxicity and gene expression analysis [18,23].

Thus, in this study we utilized human ES cell derived embryoid bodies to test efficacy of MiADMSA against arsenic poisoning. The rationale of the study was mainly to elucidate (a) if MiADMSA and arsenite showed signs of developmental toxicity per se in developing human embryoid bodies (b) does concomitant treatment of human EBs with MiADMSA and arsenic prevent arsenic-induced cell death (c) could simultaneous administration of MiADMSA prevent or reverse arsenic-induced altered gene expression and (d) could these *in vitro* results be comparable with *in vivo* studies.

2. Material and methods

2.1. Chemicals and reagents

Monoisoamyl 2,3-dimercaptosuccinic acid (MiADMSA) was synthesized in the synthetic chemistry division of our establishment, as described previously [15]. Meso 2,3-dimercaptosuccinic acid (DMSA), sodium arsenite was obtained from E. Merck (Germany). All other analytical laboratory chemicals and reagents were purchased from E. Merck (Germany) or Sigma (USA).

2.2. Cell lines and culture conditions

The human ES cell line, Relicell®hES1 used in the study was derived with proper consent and after approval from the Institutional Ethical Committee (IEC). Cells between passages no. 40 and 65 were used in the study for all the experiments. The properties and culture conditions of the cell line have been reported elsewhere [24]. Briefly, Relicell®hES1 was cultured on feeder layer of inactivated primary mouse embryonic fibroblast. The human ES culture medium used in this study consisted of 80% DMEM/F-12 (Invitrogen, CA, USA), 15% ES tested FBS (Hyclone, UT, USA), 5% serum replacement (Invitrogen, CA, USA), 1% non-essential amino-acid solution (Invitrogen, CA, USA), 1 mM glutamine (Invitrogen, CA, USA), 0.1% β-mercaptoethanol (Sigma, MO, USA), 4 ng/ml human basic fibroblast growth factor (bFGF; R&D Systems, MN, USA) and penicillin-streptomycin (Invitrogen, CA, USA). Human ES cells were maintained at 37 °C under 5% CO_2 and 95% humidity and were routinely passaged every 5–6 days.

Human foreskin fibroblast (HFF, SCRC-1042) was obtained from ATCC and was maintained in DMEM (Invitrogen, CA, USA) with 10% FBS (Hyclone, UT, USA). Cultures were maintained at 37 °C under 5% CO_2 and 95% humidity and were routinely passaged every 3–4 days.

2.3. Formation of embryoid bodies (EBs)

Human ES cells were differentiated as EBs as reported previously [23]. Briefly, EB media constituted of human ES media without bFGF. Human ES cells were trypsinized and single cell suspension made in EB media. These cells were mixed with different doses of drugs as mentioned below and placed in the lid of 85 mm petri-plate (Laxbro, India) such that each droplet of 20 μl contained 5000 cells. After cultivation of EBs in hanging drop for 4 days at 37 °C under 5% CO_2 and 95% humidity, the EBs were transferred from the hanging drop to a 60 mm low adhesion culture dishes (Nunc, Denmark) for the next 3 days (day 7) with appropriate concentration of test compound. Next, the EBs were plated separately on 0.1% gelatin (Sigma, MO, USA) coated wells of a 96 well (for cytotoxicity assay) and 6 well (for gene expression analysis) plate (containing appropriate concentration of test compound) to allow adherence and differentiation for another 8 days (day 15). Media was changed every 3 days along with appropriate concentration of the drugs. On day 15th of differentiation, cells were collected for gene expression studies from the 6 well plates whereas cytotoxicity was performed on the 96 well plates.

2.4. Drug doses and treatment

Cells were treated with log doses of the compounds, sodium arsenite and MiADMSA at concentrations of 10^3 to 10^{-6} μg/ml and 10^3 to 10^{-3} μg/ml, respectively. MiADMSA was dissolved in 3% bicarbonate solution and was added to the cell within 15 min of preparation. All further serial dilutions were made in media.

2.5. RNA extraction and quantitative real time polymerase chain reaction

RNA was extracted from the control as well as different drug treated groups using the RNeasy Kit (Qiagen, USA). Two microgram of RNA was converted to cDNA using superscript reverse transcriptase (Invitrogen, USA). Pre-designed Assay on Demand TaqMan® probes and primers were obtained from Applied Biosystems (USA). Quantitative RT-PCR analysis was conducted using ABI 7900HT Fast Real Time System (Applied Biosystems, CA, USA). After an initial denaturation cycle of 50 °C for 2 min, 94.5 °C for 10 min followed by 40 cycles of (90 °C for 30 s, 59.7 °C for 1 min). Relative changes in the gene expression were normalized with 18 s rRNA levels to calculate fold change. The details of the genes used in the study are provided in Supplementary Table 1.

2.6. Cytotoxicity assessment

Human foreskin fibroblast (HFF), human embryonic stem cell (hES) and human embryoid bodies (hEB) cytotoxicity assessment was performed using CyQUANT® cell proliferation assay kit (Invitrogen, CA, USA) as reported previously [23]. Briefly, HFF cells ($n = 200$) were plated per well and treated with or without drugs for 15 days, whereas human ES cells ($n = 1000$) were plated on 1% matrigel (BD Biosciences, CA, USA) coated plates with high bFGF concentration per well and treated with or without the drugs for 15 days. Similarly, human EB ($n = 10$) were formed and treated as mentioned above with or without drugs

Table 1
Inhibitory concentration (IC_{50}) values of the 3 cell types following arsenic or MiADMSA administration.

	HFF- IC_{50}	hES – IC_{50}	hEB – IC_{50}
Arsenic	0.3131 ± 0.01	0.6753 ± 0.02	0.00599 ± 0.00
MiADMSA	3.1506 ± 0.32	6.6003 ± 0.45	3.682 ± 0.12

Values are expressed μg/ml. The values have been calculated from the mean of three independent experiments.

for 15 days. The media was changed every 2 day with the respective drug concentration in all the three cell types. After the completion of the experiment (day 15), the media was aspirated out of the wells, cell washed once with PBS and the plates were placed in −70 °C overnight. Next day, the plates were taken out and buffer containing the CyQuant GR dye added to cells as per manufacturer's instructions. CyQuant GR dye exhibits strong fluorescence enhancement when bound to cellular nucleic acids. The fluorescence intensities emitted are proportional to the number of cells in the sample. Fluorescence intensities were measured using FluostarOptima multilabel detection system (BMG Technologies, Germany) at excitation 480 nm and emission 520 nm. The percentage viability at each test concentration was expressed based on the fluorescence, where the control (untreated) fluorescence was considered as 100% viable cells and 50% inhibitory concentration were calculated from the concentration-response curve (IC_{50}-HFF for HFF cells, IC_{50} hES for hES cells and IC_{50} hEB for hEB, respectively).

2.7. Animal studies

Forty female Wistar rats weighing 120–150 g were housed in stainless steel cages in an air-conditioned room. Rats were allowed standard rat chow diet (Lipton's India Ltd., metal contents of diet, in ppm dry weight Zn 45, Cu 10, Mn 55, Fe 70, Co 5) and water *ad libitum* for the duration of the experiment. Following acclimatization female rats and their sexually mature male rats weighing approximately 150 g, were caged for breeding in a 2:1 ratio. The day on which vaginal plugs were observed and confirmed by microscopic examination of sperms in the vaginal smear was denoted as day 0 of gestation. Each pregnant rat was kept individually in a separate cage and the cages were kept in a room with alternating light and dark cycle of 12 h each. Animal Use Ethical Committee of DRDE approved all the protocols for the experimentation. Pregnant animals were treated from day 1 to day 20 of gestation period as follows:

Group 1 – control animals, 3% sodium bicarbonate, orally
Group 2 – arsenic (III) as sodium arsenite, 0.1 mg/kg in drinking water
Group 3 – MiADMSA, 50 mg/kg, orally
Group 4 – arsenic (III), 0.1 mg/kg in drinking water + MiADMSA, 50 mg/kg, orally

The doses were selected based on previously published reports by our group [14,25]. On day 20 of gestation, the dams were euthanized and gross pathology conducted. All foetuses were examined for both soft and skeletal tissue and arsenic levels.

2.8. Elemental analysis

The arsenic concentrations in tissues and urine were measured after wet acid digestion using a microwave digestion system (Anton Paar Multiwave 3000, Austria). Samples were brought to a constant volume, and determination of tissue arsenic was

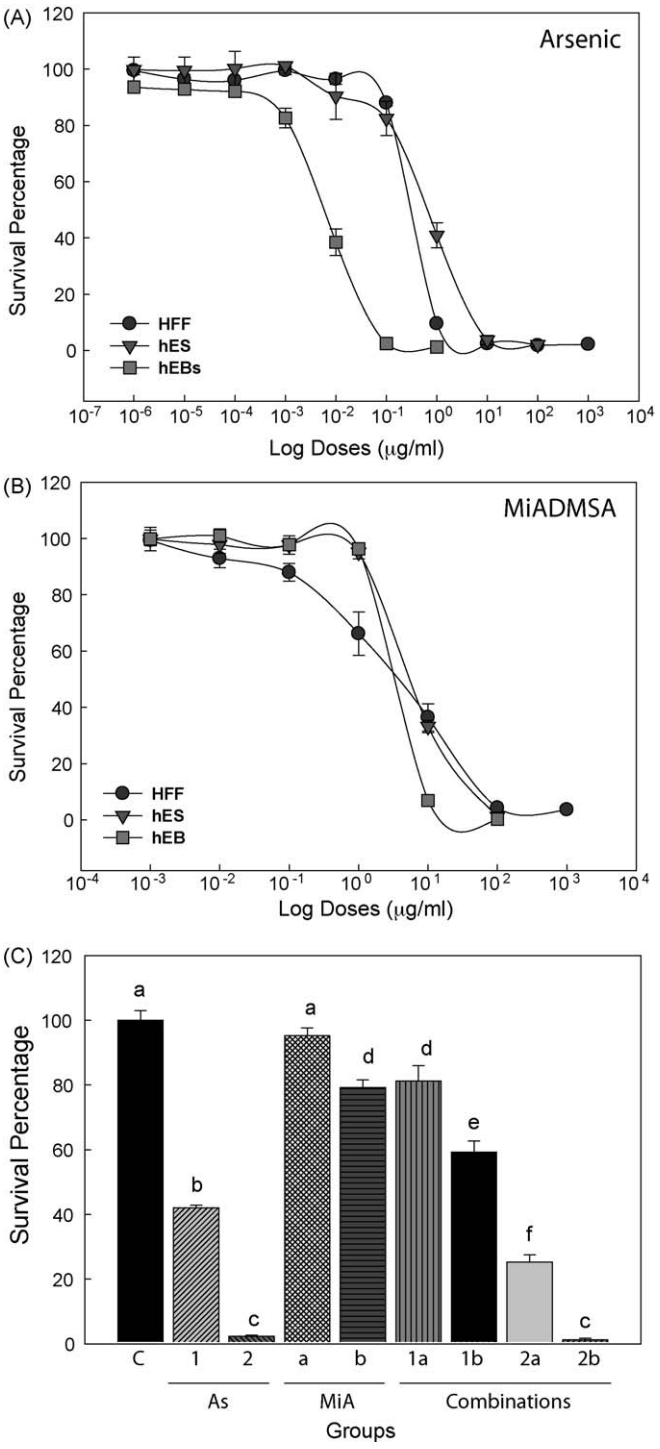

Fig. 1. MiADMSA prevents arsenic (III) induced cytotoxicity. Dose dependent cytotoxic effects of arsenic (III) (A) and MiADMSA (B) on human foreskin fibroblast (HFF), human embryonic stem cells (hES) and human embryoid bodies (hEBs) after 15 days of treatment. Cells were treated with arsenic (III) as sodium arsenite at log doses ranging from 10^{-6}–10^{3} μg/ml whereas MiADMSA at 10^{-3}–10^{3} μg/ml. C, Graph represents survival percentage of cells following treatment of arsenic (III) and MiADMSA alone or in combination on human embryoid bodies for 15 days. Cells were treated with two selected doses of arsenic (0.01 μg/ml and 0.1 μg/ml) and MiADMSA (0.1 μg/ml and 1 μg/ml) alone or in combination to evaluate the efficacy of MiADMSA in preventing arsenic-induced cytotoxicity. Abbreviations: (1) arsenic at a dose of 0.01 μg/ml; (2) arsenic at a dose of 0.1 μg/ml; (a) MiADMSA at a dose of 0.1 μg/ml; (b) MiADMSA at a dose of 1 μg/ml; 1a, 1b, 2a and 2b denotes the various dose combinations of arsenic and MiADMSA treated to human embryoid bodies. Values are mean ± S.E of three independent experiments. [a–f] Differences between values with matching symbol notations within each column are not statistically significant at 5% level of probability.

performed using an auto sampler (AS-72) and graphite furnace (MHS) fitted with an atomic absorption spectrophotometer (AAS, PerkinElmer model AAnalyst 100) as described earlier [4].

2.9. IR spectroscopy

An equimolar solution of arsenic and MiADMSA were prepared in 5% sodium bicarbonate solution and were mixed in stoichiometric ratio to perform the IR spectroscopy. Briefly, both the solutions were mixed and incubated for 1 h at 37 °C. After cooling IR spectra of individual and combined solution of arsenic and MiADMSA were recorded using PerkinElmer FTIR Spectrometer, model BXII. The spectra were recorded under the range of 4000–400 cm^{-1} with resolution of 4 cm^{-1}.

2.10. Statistical analysis

Paired comparisons were conducted using a paired *t*-test, and all data are presented as mean values ± S.E. Differences were considered significant at a 0.05 level of confidence.

3. Results

3.1. MiADMSA prevents arsenic (III) mediated cytotoxicity on human embryoid bodies

Log dose dependent treatment of arsenic (10^3 to 10^{-6} μg/ml) and MiADMSA (10^3 to 10^{-3} μg/ml) showed typical sigmoid survival curve with all the three cell type studied (Fig. 1A and B). A significant increase in cell death was observed fluorometrically at higher doses of arsenic (III) (Fig. 1A) and MiADMSA (Fig. 1B). Further, on comparison it was observed that arsenic exposed EBs were more susceptible to the toxic insult than the human foreskin fibroblast (Fig. 1A and B). On the basis of the growth curve the inhibitory concentrations (IC_{50}) were calculated for each cell type as depicted in Table 1.

On the basis of the growth survival curve, two doses of arsenic (0.01 μg/ml (As1) and 0.1 μg/ml (As2)) and MiADMSA (0.1 μg/ml (MiA1) and 1 μg/ml (MiA2)) were selected to check if concomitant administration of MiADMSA along with arsenic could prevent cell death. Co-administration of MiADMSA at lower doses (MiA1) with

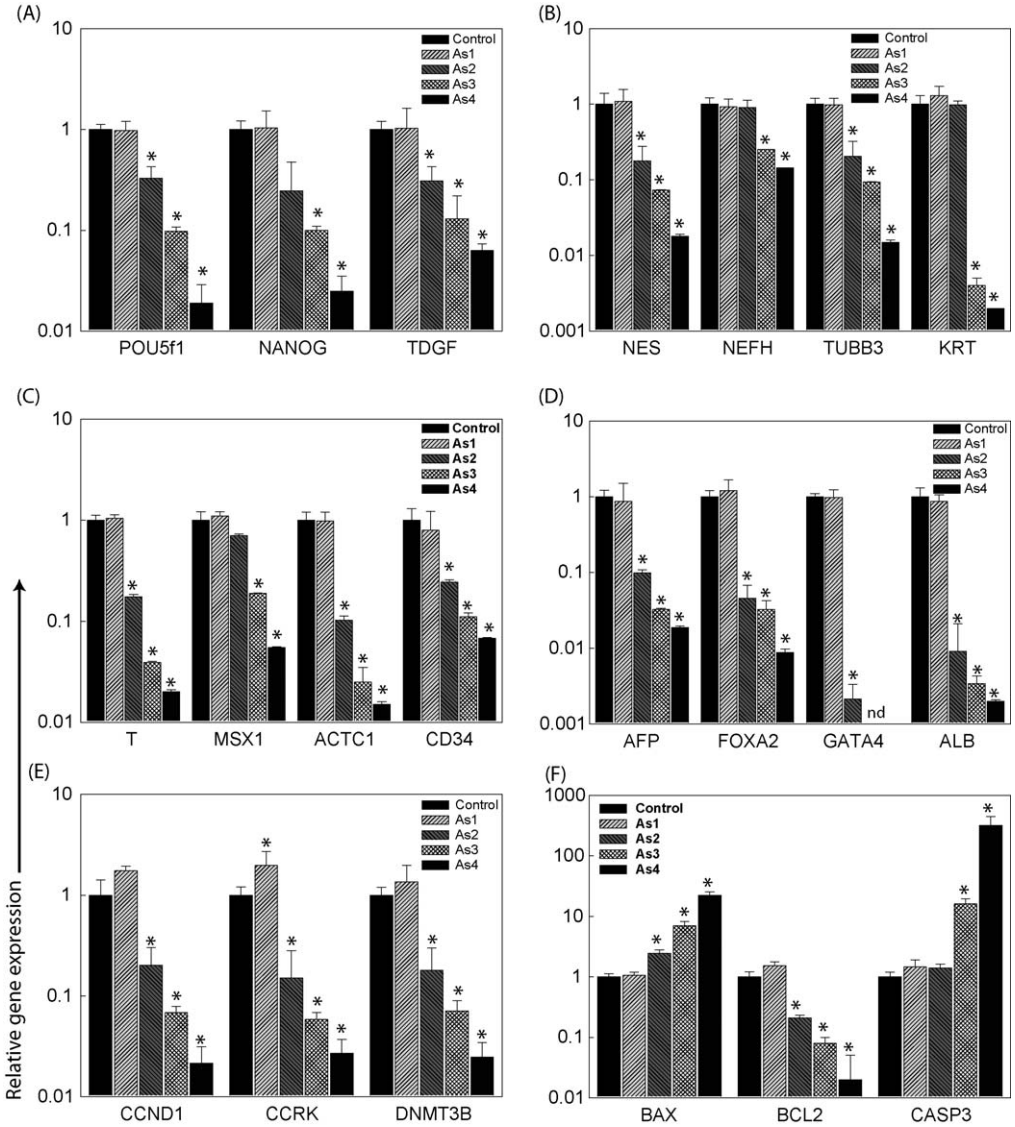

Fig. 2. Arsenic-induced altered gene expression. Graphs depicts altered relative gene expression in markers representative of pluripotency (A), ectoderm lineage (B), mesoderm lineage (C), endoderm lineage (D), cell cycle (E) and apoptosis (F) following dose dependent treatment of arsenic for 15 days on human embryoid bodies. Groups – Control, As1 – arsenic 10^{-5} μg/ml, As2 – arsenic 10^{-4} μg/ml, As3 – arsenic 10^{-3} μg/ml and As4 – arsenic 10^{-2} μg/ml. Values are mean ± S.E of three independent experiments. $^{*}P < 0.05$ is considered statistically significant. nd: not detected.

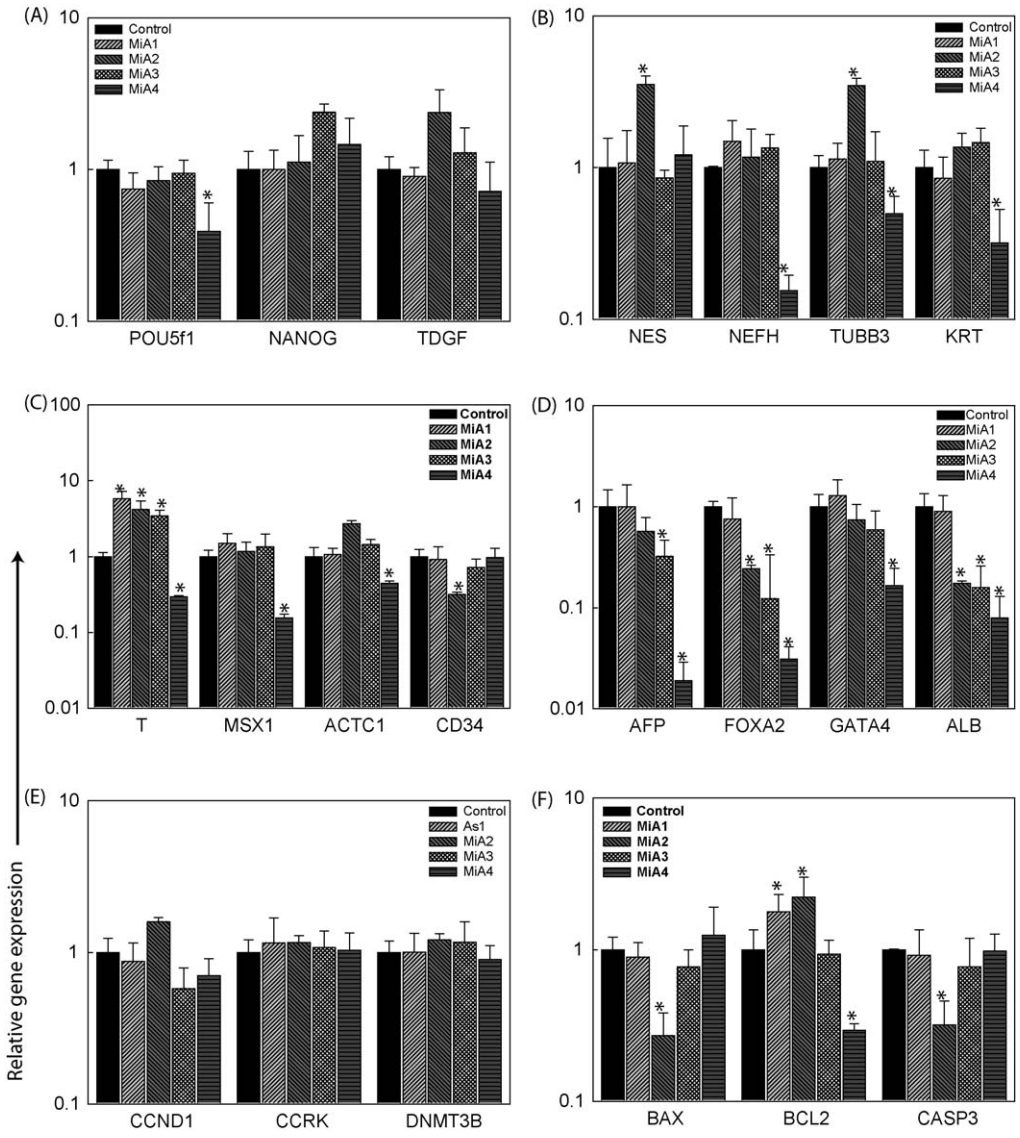

Fig. 3. Dose dependent effects of MiADMSA on gene expression. Graphs depicts altered relative gene expression in markers representative of pluripotency (A), ectoderm lineage (B), mesoderm lineage (C), endoderm lineage (D), cell cycle (E) and apoptosis (F) following dose dependent treatment of MiADMSA for 15 days on human embryoid bodies. Groups- Control, MiA1-MiADMSA 10^{-3} μg/ml, MiA2-MiADMSA 10^{-2} μg/ml, MiA3-MiADMSA 10^{-1} μg/ml and MiA4-MiADMSA 1 μg/ml. Values are mean ± S.E of three independent experiments. $^{*}P < 0.05$ is considered statistically significant.

either dose of arsenic showed significant cell survival, fluorometrically (As1 + MiA1 – 81% vs. As1 – 40%; As2 + MiA1 – 22% vs. As2 – 3%) (Fig. 1C), whereas increasing the dose of MiADMSA (MiA2) showed lesser efficacy, in terms of survival percentage (As1 + MiA2 – 62% vs. As1 – 40%; As2 + MiA2 – 2% vs. As2 – 3%) (Fig. 1C).

3.2. Arsenic (III) and MiADMSA induced altered gene expression

In order to understand the toxicity of arsenic and MiADMSA, we selected 47 marker genes representative of the undifferentiated state, three germ layers and ten derivative organs along with cell cycle, apoptosis and signaling molecules, which could provide us information about the affected lineages. Arsenic treatment significantly down-regulated (between 2 and 100 fold) most of the genes studied (Fig. 2): pluripotency (POU5F1, NANOG, TDGF1) (Fig. 2A), ectoderm (NES, NEFH, TUBB3, KRT) (Fig. 2B), mesoderm (T, MSX1, ACTC1, CD34) (Fig. 2C) and endoderm (AFP, FOXA2, GATA4 and ALB) (Fig. 2D). Apart from affecting the germ layers, significant decrease in expression was observed for genes of cell cycle (CCND1, CCRK) (Fig. 2E) and increased expression of BAX and CASP3 (apoptotic markers) (Fig. 2F). Moreover, most of these significant down-regulations in gene expression could be observed by at a dose 50 times lower than the IC_{50} values (supplementary Table 2). On the other hand per se administration of MiADMSA did not show any significant down regulation of gene expression, except at a dose of 1 μg/ml. Minimal down regulation was observed in pluripotency and ectodermal markers (Fig. 3A and B) but most of the endodermal markers (AFP, ALB, FOXA2) (Fig. 3D) suggesting its toxicity towards endodermal lineages (supplementary Table 3). No significant down regulation was seen in cell cycle regulators and apoptotic marker (Fig. 3E and F).

3.3. Reversal of altered gene expression of arsenic (III) by MiADMSA

One of the main objectives of the study was to utilize the human EB model to test the efficacy of MiADMSA against arsenic. When human EBs were simultaneously treated with MiADMSA (0.1 μg/ml) and arsenic (0.01 μg/ml) significant improvement in gene

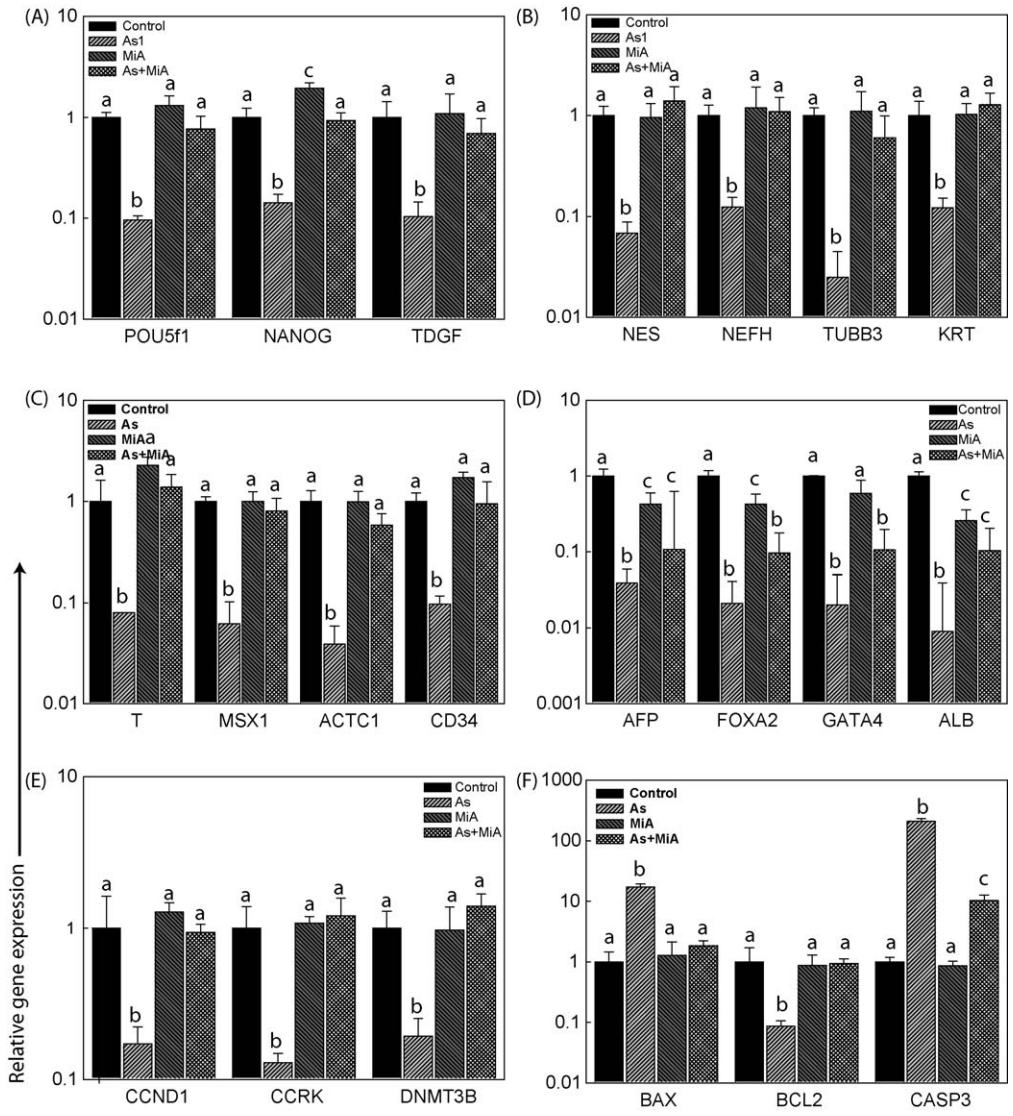

Fig. 4. Reversal of arsenic-induced gene expression by MiADMSA. Graphs depicts altered relative gene expression in markers representative of pluripotency (A), ectoderm lineage (B), mesoderm lineage (C), endoderm lineage (D), cell cycle (E) and apoptosis (F) following dose dependent treatment of arsenic and MiADMSA alone or in combination on human embryoid bodies after 15 days of treatment. Groups- Control, As -arsenic at a dose of 0.01 μg/ml, MiA-MiADMSA at a dose of 0.1 μg/ml and As + MiA-arsenic at a dose of 0.01 μg/ml + MiADMSA at a dose of 0.1 μg/ml. Values are mean ± S.E of three independent experiments. [a–c] Differences between values with matching symbol notations within each column are not statistically significant at 5% level of probability.

expression for pluripotency markers (Fig. 4A), ectodermal (Fig. 4B) and mesodermal (Fig. 4C) markers was observed, which could co-relate with the increased survival percentage (Fig. 1C). However, improvements in the gene expression for the endodermal marker was limited (Fig. 4D). Reversal of altered gene expression was also seen in cell cycle (Fig. 4E) and apoptotic markers (Fig. 4F) (Supplementary Table 4).

3.4. Efficacy of MiADMSA against arsenic in vivo

In order to validate the results generated by the human ES–EB model, we treated female Wister rats with arsenic (III) and MiADMSA alone or in combination and observed that there was a significant reduction in the litter size after arsenic treatment (number of pups per group, control-70 vs. As-30) but no significant difference was observed in MiADMSA treated group. Interestingly, animals co-administered arsenic and MiADMSA showed a significant increase in the litter size as compared with the arsenic treated group (number of pups per group, As + MiADMSA-65 vs. As-30) (Table 2). This provided some indication that MiADMSA prevented arsenic-induced death in the developing fetuses. These general observations were further supported with an increased incidence of visceral and skeletal defects after arsenic treatment. These defects were marked prevented in administered MiADMSA with arsenic (Table 2).

Further, there was a significant increase in urinary arsenic excretion in lactating dams administered MiADMSA along with arsenic (Fig. 5A), suggesting MiADMSA was capable of removing arsenic from lactating dams. Interestingly, a remarkable reduction in tissue (liver, kidney and brain) arsenic levels was also observed in pups in animals treated with MiADMSA along with arsenic (Fig. 5B).

3.5. Arsenic binds to MiADMSA via sulfhydryl as well as carboxyl groups

In order to remove arsenic from tissues or cells, binding of MiADMSA to arsenic is very important. IR spectroscopy was utilized to determine which bonds were involved in binding

Table 2
Summary of visceral and skeletal examination of foetus.

S. no.	Observation		Control	Inc (%)	As	Inc (%)	MiA	Inc (%)	As + MiA	Inc (%)
	No. of foetus		70		30	42.8	79	112	65	92.8
1	Dilated lateral ventricles – brain-grade 1		3	4.29	5	16.67	2	2.53	4	6.15
2	Dilated and fragile ventricles-brain-grade 3		2	2.86	6	20.00*	2	2.53	1	1.54†
3	#2 with dilated neural canal, small spinal cord		1	1.43	6	20.00*	–	0	–	0
4	#1 with fragile and ruptured cerebral hemisphere-grade 2		–	0	5	16.67	–	0	–	0
5	Brownish discoloration around cerebral hemisphere – grade 1		–	0	6	20.00*	–	0	2	3.08†
6	Hemorrhagic foci – grade 1		–	0	7	23.33*	–	0	4	6.15†
7	Subcutaneous hemorrhages – grade 1		1	1.43	2	6.67	1	1.27	1	1.54
8	Yellowish pervascular area- liver- grade 3		1	1.43	9	30.00*	3	3.80	9	13.85†
9	Anophthalmia – unilateral		–	0	4	13.33	–	0	2	3.08
10	Microhphthalmia – unilateral		1	1.43	5	16.67	1	1.27	5	7.69
11	Microhphthalmia – bilateral		–	0	10	33.33*	2	2.53	4	6.15†
12	Small renal papillae		1	1.43	9	30.00*	3	3.80	6	9.23†
13	Absent renal papille		–	0	3	10.00	–	0	–	0
14	Absent renal papille – unilateral		1	1.43	2	6.67	1	1.27	1	1.54
15	Dilated renal pelvis – grade 1		–	0	4	13.33	3	3.80	4	6.15
16	Dilated renal pelvis – grade 2 and 3		–	0	5	16.67	2	2.53	3	4.62
17	Dilated renal pelvis – unilateral		–	0	3	10.00	–	0	2	3.08
18	Brownish discoloration – lung grade 4		2	2.86	6	20.00*	2	2.53	1	1.54†
19	Greenish discoloration – lung grade 1		1	1.43	2	6.67	–	0	–	0
20	Sternum	Manubrium	1	1.43	7	23.33*	2	2.53	1	1.54†
		Sternbrae	7	10.00	20	66.67*	12	15.19	19	29.23†
		Xipisternum	20	28.57	25	83.33*	24	30.38	25	38.46†
21	Number of vertebrae	Thoracic	–	0	12	40.00*	5	6.33	10	15.38†
		Lumbar	1	1.43	4	13.33	3	3.80	9	13.85
		Sacrum	1	1.43	2	6.67	–	0	–	0
22	Cranial skeleton	Absent mandible	–	0	5	16.67	–	0	6	9.23
		Absent Tympanic ring	–	0	3	10.00	3	3.80	2	3.08
		Widen anterior fontanellae	2	2.86	7	23.33*	5	6.33	3	4.62†
		Widen fontanellae and small holes in parietal and interpariertal	1	1.43	4	13.33	–	0	–	0
		Widen naso-frontal suture, fontanellae	2	2.86	2	6.67	3	3.80	1	1.54
		Widen naso frontal suture	1	1.43	5	16.67	2	2.53	1	1.54
23	Axial skeleton	Absent ribs	2	2.86	5	16.67	2	2.53	3	4.62
		Bend and wavy ribs	–	0	8	26.67*	4	5.06	3	4.62†
		Short ribs	2	2.86	5	16.67	–	0	2	3.08
		Bilobed centrum	–	0	3	10.00	–	0	–	0
		Small sterenebra	1	1.43	1	3.33	1	1.27	1	1.54
		absent venteral arch	–	0	–	0	1	1.27	2	3.08
24	Limbs	Absent/short pubis	2	2.86	7	23.33*	2	2.53	3	4.62†
		Broken radius	1	1.43	5	16.67	2	2.53	1	1.54
		Fusion- radius – ulna	2	2.86	6	20.00*	3	3.80	4	6.15†
		short ischium	–	0	2	6.67	–	0	1	1.54
25	Incomplete/delayed ossification	Parietal	12	17.14	20	66.67*	14	17.72	21	32.31†
		Interparietal	10	14.29	14	46.67*	15	18.99	12	18.46†
		Supro-occipital	3	4.29	8	26.67*	3	3.80	1	1.54†
		Skull	2	2.86	8	26.67*	4	5.06	3	4.62†
		Mandible	1	1.43	4	13.33	1	1.27	1	1.54
		Sternabrae	2	2.86	5	16.67	5	6.33	2	3.08
		Ribs	1	1.43	2	6.67	1	1.27	2	3.08
		Frontal	–	0	4	13.33	1	1.27	1	1.54
		Pubis	1	1.43	6	20.00*	2	2.53	1	1.54†
		Xipisternum	2	2.86	7	23.33*	2	2.53	5	7.69†

Abbreviations: S. no.– serial number; Inc% – Incidence percentage; As: arsenic treated group; MiA-MiADMSA treated group; As + MiA – arsenic and MiADMSA treated group. Statistical analysis was performed using Student's *t*-test.

* $p < 0.05$ compared to controls.

† $p < 0.05$ compared to arsenic treated group.

MiADMSA to arsenic. The spectra of arsenic showed only two sharp peaks (1650.20 and 1408.67 cm^{-1}) (Fig. 6B) similar to the vehicle (sodium bicarbonate) (Fig. 6A). On the other hand, MiADMSA gave a weak absorption in the range of 2200–2300 cm^{-1} (Fig. 6C) while, the combined spectra of arsenic and MiADMSA showed doublet at the same position (Fig. 6D). Apart from this MiADMSA shows C=O stretching of the free carboxylic acid group at 1647.67 cm^{-1} (Fig. 6C) which was shifted towards lower frequency side in combination (Fig. 6D). A single peak at 1384.04 cm^{-1} in MiADMSA alone was observed while in combination two different peaks were obtained at 1464.68 cm^{-1} and 1279.77 cm^{-1} (Fig. 6D), indicating binding of the two molecules. Further, the sharp peaks were observed at 801.48 cm^{-1} and 667.7 cm^{-1} suggesting binding of arsenic to MiADMSA (Fig. 6D).

4. Discussion

Arsenic is a well known slow poison, known to affect all major organ functions in human. Arsenic poisoning through contaminated drinking water has been reported from various parts of the

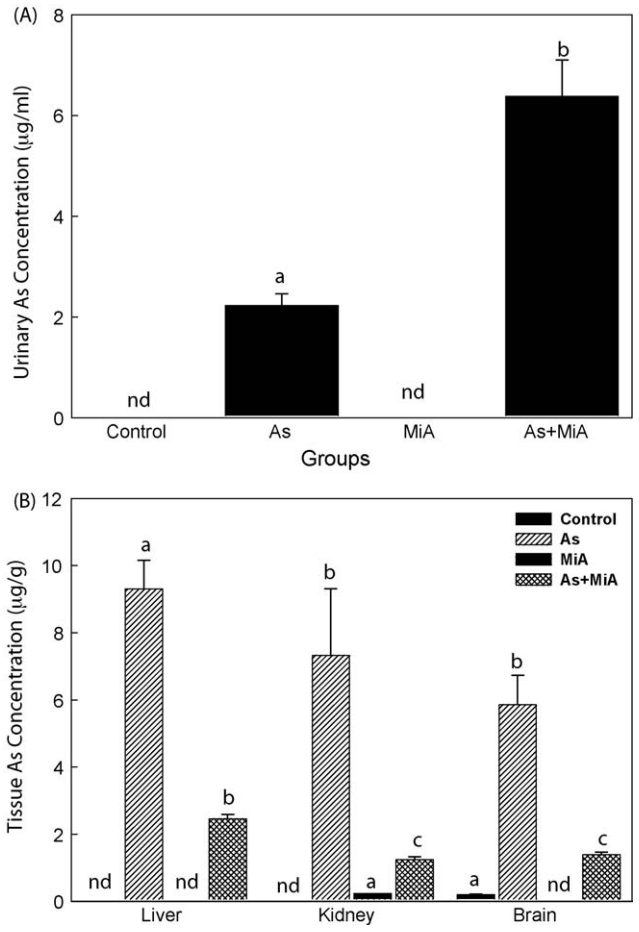

Fig. 5. MiADMSA increase urinary excretion of arsenic. (A) Graph depicts increased urinary excretion in pregnant animals treated with MiADMSA along with arsenic. Animals were treated with 0.1 mg/kg dose of arsenic (III) and MiADMSA (50 mg/kg) either alone or in combination. Data represents mean ± S.E of five animals. [a-c] Differences between values with matching symbol notations within each column are not statistically significant at 5% level of probability. (B) Tissue arsenic concentration in major organs (liver, kidney and brain) of pups. Data represents mean ± S.E of randomly selected 10 pups from each group. [a-f] Differences between values with matching symbol notations within each organ are not statistically significant at 5% level of probability.

world but has its biggest centre in the Indo-Bangladesh region where millions of people are daily consuming water with high arsenic levels (up to 3000 ppb). Chelation therapy is the only respite for these people to reduce the toxic burdens of arsenic. However, placebo trials have shown that DMSA was not effective in reducing arsenic burdens in these patients [11]. One of the probable reasons for this failure could be attributed to the hydrophilic nature of DMSA. In order to address this issue, our group in the last decade worked on various strategies including development of mono- and di-esters analogues of the parent drug DMSA, to increase its lipophilicity. We found MiADMSA (C_5-branched mono-isoamyl derivative of DMSA) to be highly effective in not only reducing heavy metal burdens in animal models from various organs but also providing better recoveries in numerous biochemical and molecular variables in animal models [4,13,15,16,26].

Human embryonic stem cells have added a new dimension to already existing tools in toxicology [20]. The ability of human ES cells to form three germ layers and ability to differentiate into various cell types after induced differentiation could cater to the ever growing needs of the pharmaceutical industry to screen compounds for better and safer drug molecules for human consumption. Recently, we for the first time reported that the human ES–EB model could be used to evaluate pharmaceutical compounds for its developmental toxicity. However, the study has its own limitation as it only provided information about the developmental toxicity of the tested compound but could not provide any information on the efficacy of the drugs [23]. Thus, the present study was planned to evaluate the efficacy of MiADMSA against arsenic (III) in the human ES–EB model and to compare its results with the *in vivo* model.

Our preliminary data suggested that when arsenic (III) is treated with MiADMSA, survival of human EBs increased by 40% *in vitro* (Fig. 1C). Similarly co-administration of MiADMSA with arsenic in the pregnant rats increased the number of surviving pups by 50% (Table 2), providing the first evidence of efficacy for MiADMSA against arsenic-induced mortality. We also observed that human ES cell derived embryoid bodies were more sensitive to toxic insults that human foreskin fibroblast. One of the reasons for this difference could be that fibroblast are mature and terminally differentiated cell types where as embryoid bodies, although have made commitment towards differentiation by the formation of the three germ layers but are comparatively immature and hence more sensitive to toxic insults as compared with foreskin fibroblasts. Further, we carried out gene expression studies from human EBs to check which germ lineages or specific cell types are being affected by arsenic and MiADMSA alone. It was observed that cells from all the three germ layers were affected by arsenic whereas MiADMSA showed mild toxic effects to endodermal cell. This observation was in concordance with our previous studies where administration of MiADMSA caused mild temporary hepatotoxic effects in animal models but these effects subsided once the drug administration was terminated [25,27–29]. We also previously reported that MiADMSA and DMSA when administered alone to pregnant rats from gestation to lactation did not show significant alterations in gestation, litter-size, sex ratio, viability and lactation. Further no skeletal defects were observed except a loss of copper [25]. In the present study, visceral and skeletal examinations of pups showed lesions in most of the organs treated only with arsenic (III) and not MiADMSA (Table 2).

Although initial data suggested that MiADMSA prevented developmental cytotoxic effects of arsenic (III) but does MiADMSA have the ability to reverse arsenic-induced altered gene expression, which would logically result in better clinical recoveries needed to be evaluated. Most of the gene expression altered by arsenic treatment could be reversed to various extends following concomitant administration of MiADMSA with arsenic (III) in human embryoid bodies (Fig. 4). Similar results too were observed in the *in vivo* model, where the percentage incidences of lesions were significantly reduced when MiADMSA was administered along with arsenic (III) (Table 2). Overall results generated by two independent models (*in vitro* and *in vivo*) clearly showed that arsenic (III) caused embryotoxic effects, which could be reversed by concomitant treatment of MiADMSA. Further, results also indicated that humane embryonic stem cell derived embryoid bodies could be utilized as an efficient model to study developmental or embryotoxicity of pharmaceutically active compounds. Although, the human ES–EB model may not provide information like visceral and skeletal defects but would effectively provide information based on gene expression about the formation of the major organs from the three germ layers.

One of the major reasons which could be attributed for the reversal of altered variable could be the removal of available arsenic by MiADMSA thus preventing arsenic-induced alterations. This was supported by an increased urinary excretion of arsenic in pregnant rats (Fig. 5A) as well as reduced arsenic concentration in major organs (liver, kidney and brain) of pups (Fig. 5B) treated with MiADMSA. In order to further validate the binding ability of MiADMSA with arsenic, IR spectroscopy was performed to evaluate

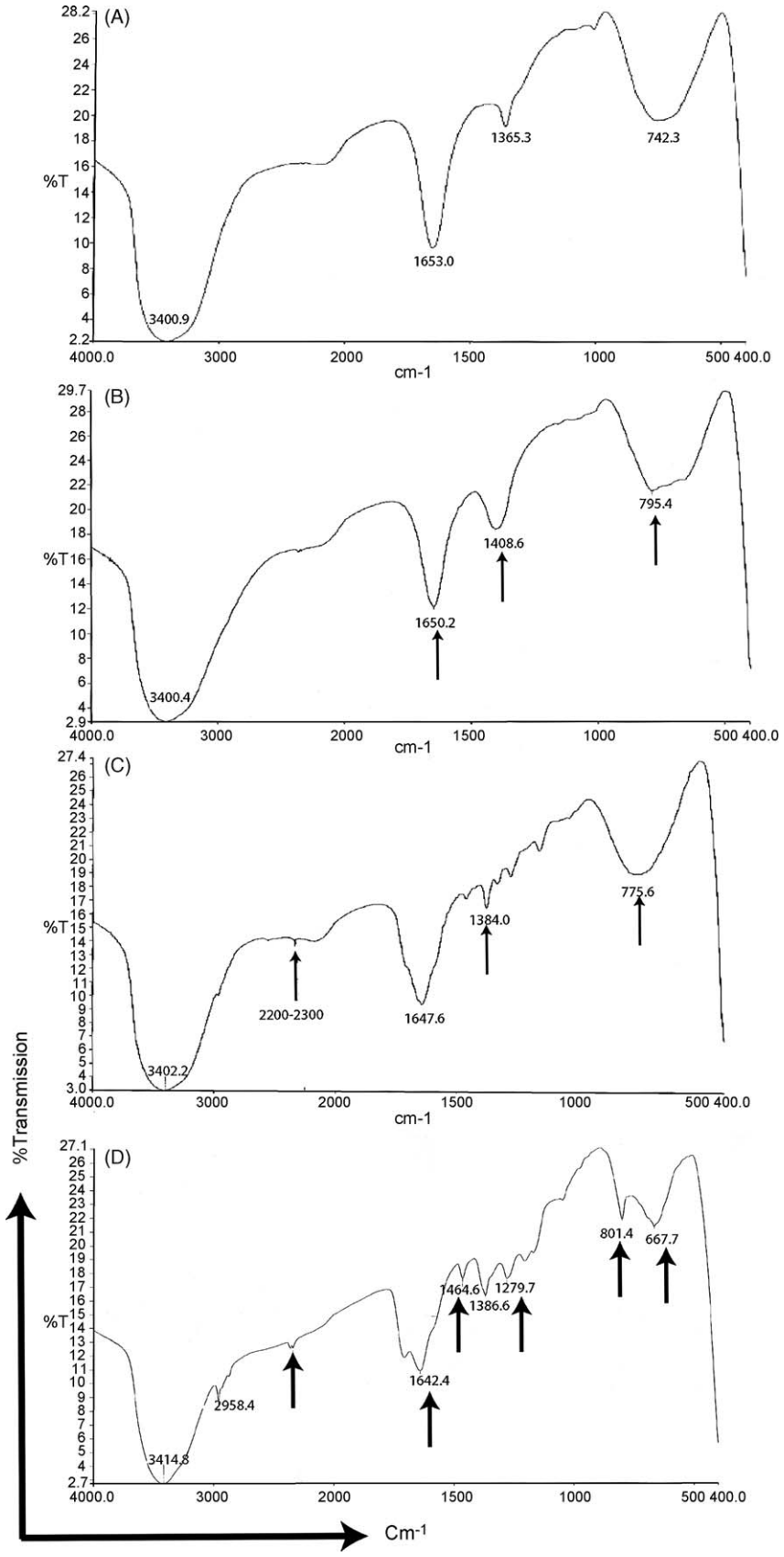

Fig. 6. IR spectroscopy shows binding of arsenic to MiADMSA. IR spectra show a significant shift in the peak intensities when arsenic and MiADMSA are mixed together. (A) Vehicle (sodium bi carbonate), (B) arsenic, (C) MiADMSA, (D) arsenic + MiADMSA. Note the shift of peaks in the combination as compared to the individual spectra. The thick arrows in spectra D denotes the important signals which indicate towards the binding of MiADMSA to arsenic.

the changes in bonding pattern, if any. Interestingly, in combination (MiADMSA + arsenic) the characteristic peak of –SH group (2200–2300 cm^{-1}) was replaced by a doublet and a shift in C=O group (1647.6 cm^{-1} to 1642.4 cm^{-1}) was noted. Formations of new peaks at 1464.6 cm^{-1} and 1279.7 cm^{-1} suggesting formation of adduct of arsenic and MiADMSA (Fig. 6), utilizing two different functional groups in chelation of arsenic with MiADMSA. Presence of sharp peaks at 801.4 cm^{-1} may be due to C–H bond of succinic acid residue of MiADMSA which could have appeared due to the distortion of MiADMSA structure due to its binding with arsenic. Lastly, the sharp peak signal at 667.7 cm^{-1} could be observed corresponding to As–O–As stretching mode. A signal at 600 cm^{-1} may be produced due to Cu–O–Cu stretching mode [30], however since arsenic is heavier than copper the signal is shifted to 667.7 cm^{-1} as observed, further confirming the binding of MiADMSA to arsenic. Interactions of heavy metals (Pb, Cd and Hg) with monomethyl ester of DMSA as initially reported by Rivera and co worker [31] and they too showed the involvement of two functional groups (one –SH and one C=O) with heavy metals.

In conclusion, this is the first proof of concept report in our knowledge where the application of human ES–EB model has been utilized to study the efficacy of a potential drug against arsenic. The study suggested that (a) the human ES–EB model is an effective model to study early developmental toxicity and efficacy of compounds; (b) concomitant administration of MiADMSA reversed arsenic-induced altered gene expressions (c) the data generated using human ES cells are highly comparable with the *in vivo* animal model; (d) the efficacy of MiADMSA against arsenic is mainly attributed due to the ability to the formation an adduct utilizing the sulfhydryl and carboxyl groups. However, the present design of the human ES–EB model could have some impedes in studying therapeutic efficacies, for which better understanding of the human ES–EB model is required.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2009.07.003.

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